Functional Characterization of the Putative RNA Helicase HELZ

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Philippe Arye Hasgall

aus

Fontainemelon (NE)

Promotionskomitee

Prof. Dr. Roland H. Wenger (Vorsitz)
PD Dr. Gieri Camenisch (Leitung der Dissertation)
Prof. Dr. Doerthe Katschinski
Dr. Thomas Gorr

Zürich, 2011

This doctoral thesis has been performed under the supervision of

Dr. Gieri Camenisch and

Prof. Dr. Roland H. Wenger

at the Institute of Physiology and Zürich Center for Integrative Human Physiology (ZIHP), University of Zürich, CH-8057 Zürich, Switzerland.

This thesis is dedicated in memory of my mother

Elisabeth Hasgall-Weill

(1939-2006)

Part 1: Summary

English

Aerobic organisms require oxygen to produce ATP, the energy unit of the cells. To ensure adequate ATP production, cells developed several different adaptation processes to meet variations in oxygen supply. Central in the molecular adaptation is the hypoxia-inducible factor HIF, a transcription factor that regulates the expression of several dozens of target genes that are involved in systemic as well as cellular adaptation processes. HIF belongs to the basic helix-loop-helix/Per-ARNT-Sim (bHLH/PAS) family of transcription factors and is composed of a constantly expressed HIF-β and an oxygen-labile HIF-α subunit [3]. The HIF-α subunit is tightly regulated by a family of HIF proly-4-hydroxylases (PHDs) that use oxygen as substrate to hydroxylate two prolines in a LxxLAP motif within the HIF-α protein enabling pVHL-mediated E3 ubiquitin ligase binding resulting in HIF-α ubiquitination and proteasomal degradation [3-5]. There are three major PHD isoforms termed PHD1, PHD2 and PHD3. PHD2 is thought to be the key regulator of HIF-α [6, 7].

In the work presented herein we attempted to find novel PHD2-interacting proteins. Using yeast 2-hybrid methodology, we discovered three novel PHD2 interactors: helicase with zinc finger domain (HELZ), zinc finger and BTB domain containing protein (ZBTB) 3 and KIAA0556. Bioinformatic analysis revealed that HELZ, similar to HIF-α, has two highly conserved LxxLAP domains. However, in contrary to HIF-α, HELZ protein levels were not regulated by the PHD function. Furthermore, hypoxia had no effect on HELZ mRNA levels as well as subcellular localization and HELZ was not involved in the regulation of HIF-transcriptional activity.

To ensure proper function, cells developed adaptation mechanisms allowing rapid response to change in their physiological environment. Protein translation plays a central role in these adaption processes, since it allows an immediate response even when gene transcription is lacking. Translation initiation, the rate limiting step of protein translation, involves many proteins including initiation factors, ribosomal

subunits and general RNA binding proteins. One key player in this regulation is the poly(A) binding protein (PABP) that binds to the poly(A) tail of all poly-adenylated mRNAs and brings about their circularization, a process that facilitates translation initiation [8, 9]. We found that HELZ associates with PABP via its PAM2 motif, a protein interaction motif found in various PABP interactors [10]. Furthermore, interaction with PABP suggested a role for HELZ in protein translation, in particular in translation initiation. Indeed, siRNA-mediated HELZ *knock down* reduced and transient overexpression of HELZ induced the translation of a heterologous luciferase gene reporter as well as general protein translation. This stimulatory function was independent of the HELZ helicase function as well as the prolines found within the HELZ LxxLAP motifs as demonstrated by site-directed mutagenesis analysis. In addition, polysomal shift analysis revealed impaired translation initiation in HELZ-silenced HeLa cells supporting the notion that HELZ functions in translation initiation. Mechanistically, we were able to show that HELZ promotes cell proliferation and most strikingly, HELZ strongly impairs ribosomal protein S6 phosphorylation without affecting S6 protein expression.

In summary, we suggest a role for HELZ as novel translation initiation factor that promotes cell proliferation as well as S6 phosphorylation.

Deutsch

Aerobe Organismen benötigen Sauerstoff um ATP zu produzieren. Um eine adäquate ATP Produktion zu gewährleisten, haben Zellen im Laufe der Evolution Anpassungsprozesse an die sich verändernde Sauerstoffverfügbarkeit entwickelt. In der molekularen Anpassung ist der Hypoxie-induzierbare Transkriptionsfaktor HIF zentral. HIF reguliert die Expression einer grossen Anzahl von Zielgenen, die in den systemischen sowie zellulären Anpassungsvorgängen eine Rolle spielen. HIF ist ein heterodimerer Proteinkomplex, der sich aus einer konstitutiv exprimierten HIF-β und einer sauerstoffregulierten HIF-α Untereinheit zusammensetzt. Die Stabilität der HIF-α Untereinheit wird von einer Familie von HIF Prolyl-4-Hydroxylasen (PHDs) reguliert. Diese Enzyme verwenden Sauerstoff als Substrat, um zwei spezifische Proline, die in einem konservierten LxxLAP Sequenzmotif im HIF-α vorkommen, zu hydroxylieren. Diese Hydroylierung ermöglicht wiederum die Bindung des von Hippel-Lindau Proteins an die HIF-α Untereinheit, was zur Ubiquitinierung und dem darauffolgenden Abbau der HIF-α Untereinheit im Proteasom führt. Die PHD Enzymfamilie besteht aus drei Hauptisoformen: PHD1, PHD2 und PHD3. Obwohl alle drei Isoformen HIF-α hydroxylieren können, deuten verschiedene Studien darauf hin, dass die PHD2 Isoform der zentrale HIF-α Regulator ist.

Mit dem Ziel weitere Interaktionspartner von PHD2 zu identifizieren, haben wir eine Hefe Zwei-Hybrid Durchforstung durchgeführt und drei noch unbekannte PHD2 Interaktoren gefunden: Die Helikase mit einer Zinkfinger Domäne (HELZ), das Zinkfinger und BTB-Domänen Protein (ZBTB) 3 und KIAA0556. Eine bioinformatische Analyse dieser neuen Interaktoren hat ergeben, dass HELZ, ähnlich zu HIF-α, zwei konservierte LxxLAP Motife enthält. Die HELZ Proteinmenge wird jedoch nicht durch die PHDs reguliert. Hypoxie hat keinen Einfluss auf die HELZ mRNA Mengen und auch nicht auf die subzelluläre Lokalisierung von HELZ. Auch hat HELZ keinen Einfluss auf die transkriptionelle Aktivität von HIF.

Um eine einwandfreie Zellfunktion sicherzustellen, haben Zellen Anpassungsmechanismen entwickelt, die eine schnelle Anpassung an sich ändernde physiologische Bedingungen ermöglicht. Die Proteintranslation spielt eine zentrale Rolle in diesen Anpassungsvorgängen, da diese eine schnelle Reaktion, sogar bei fehlender

Gentranskription, ermöglicht. Die Translationsinitiierung ist der limitierende Faktor in der Proteintranslation und umfasst eine Vielzahl von Proteinen, unter anderem Initiationsfaktoren, ribosomale Untereinheiten und RNA-Bindungsproteine. Eine Schlüsselrolle in diesem Prozess spielt das Poly(A)-Bindungsprotein (PABP), das spezifisch zum Poly(A)-Schwanz aller polyadenlylierter mRNA bindet. Diese Bindung unterstützt die Zirkularisation der mRNA, ein Vorgang, der die Translationsinitiierung unterstützt. Wir haben festgestellt, dass HELZ durch sein PAM2 Motif - ein Proteinbindungsmotif das in verschiedenen PABP-Interaktoren vorkommt - mit PABP interagiert. Diese Interaktion deutet auf eine Funktion von HELZ in der Proteintranslationsinitiierung hin. Die siRNA-vermittelte Herunterregulation von HELZ reduzierte, und die transiente Überexpression von HELZ induzierte die Expression eines heterologen Luziferase Reportergens ohne den Luziferase mRNA Pegel zu verändern, was eine Rolle von HELZ in der generellen Proteintranslation vermuten lässt. Auch wurde in Bezug auf die generelle Proteintranslation eine stimulierende Funktion von HELZ gemessen. Diese induzierende Wirkung von HELZ auf die Proteintranslation ist unabhängig von der Interaktion von HELZ mit PABP, sowie von der Präsenz der Proline in den HELZ LxxLAP Motifen. Auch bestätigte eine Polysomenshiftanalyse von HeLa Zellen, die reduzierte HELZ Proteinmengen vorweisen, eine Rolle von HELZ in der Proteintranslation.

Im Weiteren konnten wir zeigen, dass HELZ die Zellproliferation stimuliert und interessanterweise auch die Phosphorylierung des ribosomalen Proteins S6, ohne dessen Gesamtmenge zu beeinträchtigen.

Zusammenfassend schlagen wir eine Funktion von HELZ als neuen Translationsinitiationsfaktor, der das Zellwachstum sowie die Phosphorylierung des ribosomale Proteins S6 stimuliert, vor.

Part 2: Abbreviations

(A)-site	Acceptor site
(E)-site	Exit site
(P)-site	Peptidyl site
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
bHLH	basic helix-loop-helix
cAMP	cyclic AMP
CBP	CREB-binding protein
cDNA	complementary DNA
CO_2	Carbon dioxide
CREB	cAMP response element-binding protein
Da	Dalton
Dbp	DEAD box protein
DNA	Deoxyribonucleic acid
DRHC	Down-regulated in human cancer
eEF	Eukaryotic elongation factor
EGL	Egg-laying abnormal
eIF	Eukaryotic initiation factor
EPO	Erythropoietin
FIH	Factor inhibiting HIF
FKBP	FK506-binding protein
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
HELZ	Helicase containing a zinc finger
HIF	Hypoxia-inducible factor
HRE	Hypoxia-responsive element
iPABP	inducible PABP
MAPK	Mitogen-activated protein kinase
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
NMD	Nonsense-mediated decay
NTP	Nucleosid triphosphate
ODD	Oxygen-dependent degradation
PABP	Poly(A) binding protein
Pam	Poly(A)-interaction motif
PAS	Per, Arnt, Sim
PHD	Prolyl -4-hydroxylase domain-containing protein
Poly(A)	Poly adenylated
Prp	Pre-mRNA-processing protein
pVHL	Von-Hippel Lindau protein
RNA	Ribonucleic acid
RNP	Ribonucleoproteins
RRM	RNA recognition motif
rRNA	ribosomal RNA
SF	Superfamily
SIAH	Seven in absentia homolog

Abbreviations

TAD	Transactivation domain
tPABP	testis PABP
tRNA	transfer RNA
UAP	U2AF65-associated protein
UPF	Up-frameshift
UTR	Untranslated region
UvrD	UV-resistant D
IRP	Iron regulatory protein
ATF	Activating transcription factor

Part 3: Introduction

RNA helicases

The term helicase describes enzymes that unwind deoxyribonucleic acid (DNA) and Ribonucleic acid (RNA) duplexes in a nucleosidetriphosphate (NTP)-dependent fashion [11]. RNA helicases are found in viruses, bacteria, archaea and eukaryotes, where they are the largest group of enzymes involved in RNA metabolism [12]. They play a role in almost all areas of RNA processing such as messenger RNA (mRNA) translation, ribosome synthesis, RNA maturation, nuclear export, etc. [13].

One of the first described helicases was the eukaryotic translation initiation factor (eIF) 4A. In 1985, a research team lead by Nahum Sonnenberg incubated eIF4A protein together with globin mRNA and as a result observed an increase in nuclease sensitivity of the globin mRNA. This experiment suggested that eIF4A can unwind the mRNA structure. Adenosine triphosphate (ATP) was required for this reaction [14].

3.1 Classification

Helicases are identified based on the sequence similarity of seven conserved motifs called “helicase signature motifs” and are classified based on the organization and similarity of those motifs into three large superfamilies and two smaller families [15]. Superfamilies 1 and 2 (SF1 and SF2) contain all seven motifs whereas superfamily 3 (SF3) contains only three [16]. In Figure 1 the motifs of the three superfamilies with the corresponding consensus sequences are depicted.

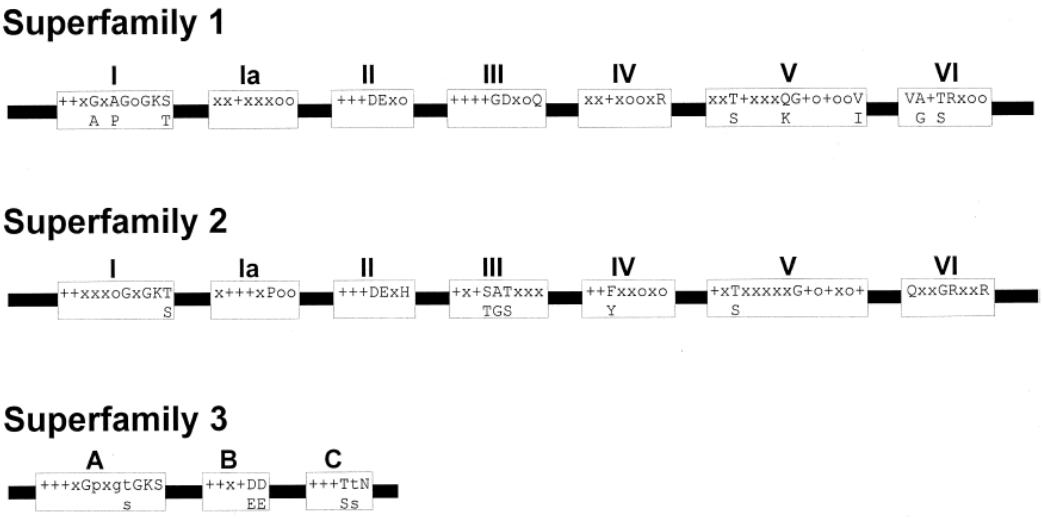


Figure 1: Schematic diagrams representing proteins from each of the three helicase superfamilies with the N-termini on the left and C-termini on the right. In all family consensus sequences, a '+' represents a hydrophobic residue, an 'o' represents a hydrophilic residue and an 'x' represents a residue that is not restricted to being hydrophobic or hydrophilic. [17]

The seven motifs of SF1 and SF2 are usually found within a region of 200–700 amino acids called the "core region" [18]. The sequence between the motifs is highly variable but the length is mostly conserved. The C- and N-terminal regions are highly variable. Most of these motifs are involved in NTP binding or hydrolysis to unwind double-stranded nucleic acid. In Figure 2 the motifs with their corresponding functions are depicted.

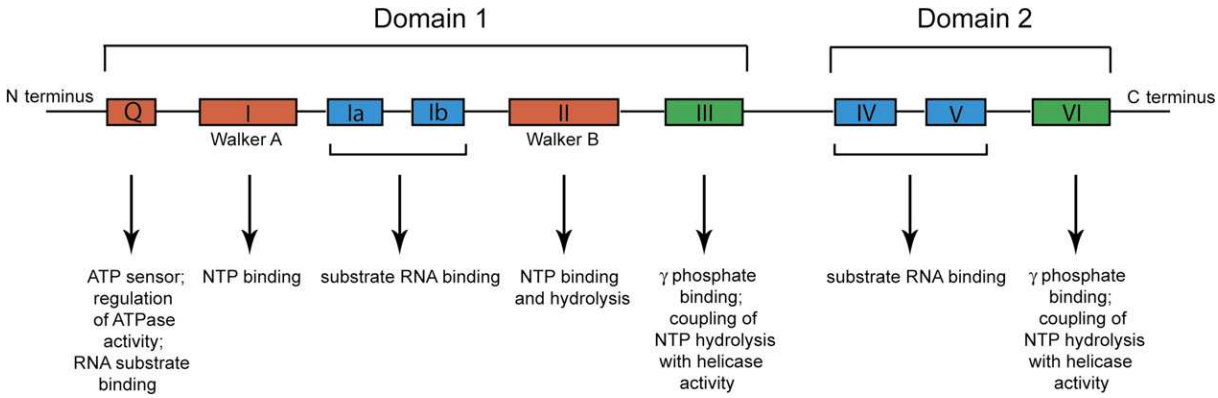


Figure 2: Conserved sequence motifs of SF1 and SF2 RNA helicases and their functions. [19]

Even though SF1 and SF2 harbor all seven helicase key motifs they are nevertheless easy distinguishable since some of their seven motifs do not share sequence similarity and they also differ in the localization of some of the motifs on the enzyme. Furthermore, sequence comparison of yeast SF1 RNA helicases revealed two additional SF1-specifc motifs and RNA helicases that contain these additional motifs may belong to a subset of the SF1 that are specifically involved in RNA-dependent processes [20].

It is important to note that the sequence comparison only allows establishing families of related proteins but do not assign a specific function to the protein. Therefore, as long as a helicase function has not been proven experimentally, the functionality of a putative RNA helicase has to be questioned.

Two motifs, that are common and highly conserved between the superfamilies, are the Walker A (phosphate-binding (P)-loop) and Walker B (Mg^{2+}-binding aspartic acid) motifs [21]. These are also found in many NTPase-containing enzymes [22].

3.1.1 **Walker A motif**

The Walker A motif is crucial for the ATPase and helicase function. For example, mutation of the first alanine residue in the Walker A region of eIF4A to valine or mutation of a conserved lysine to asparagine abolished its ATPase and unwinding activity completely [23]. The Walker A motif contains the consensus sequence (G/A)xxxxGK(T/S) (where x is any amino acid) [24].

8.1.1 **Walker B motif**

The Walker B motif contains the consensus DExx. Mutation of either the aspartic or glutamic acid residue abolishes the ATP binding and hydrolysis function [23]. The glutamic acid is thought to function as the catalytic base in the ATP hydrolysis [24].

8.1.2 **Superfamily 1**

SF1 helicases are found throughout all kingdoms even including viruses [25]. The best characterized member of this group is up-frameshift (Upf) 1, an enzyme required for nonsense-mediated mRNA decay (NMD) [26]. SF1 consists of three families: UV-resistantD/replication (UvrD/Rep), petite integration frequency 1(Pif1)-like and Upf1-like and only the Upf1-like family contains RNA helicases [1, 27] (Figure 3). This Walker B motif of SF1 includes the consensus DExx [16].

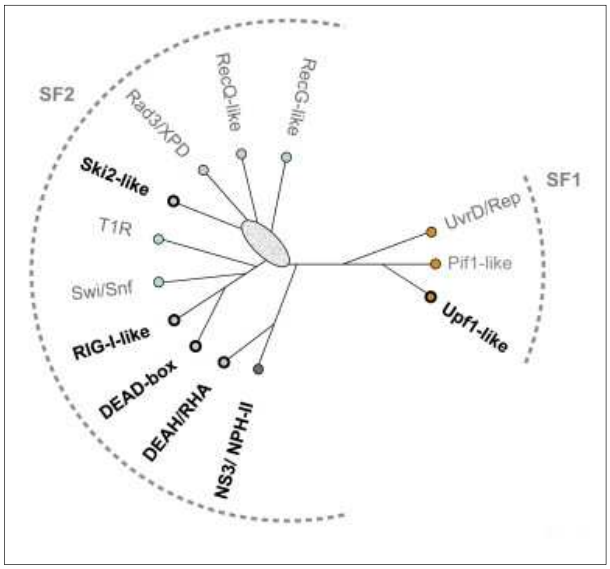


Figure 3: SF1 andSF2 helicase families. Unrooted cladogram showing the families of the SF1 and the SF2. Families containing RNA helicases are written in bold. Uvr, UV-resistant; Rep, replication; Pif, petite integration frequency; Rec, recombination; Rad, radiation; XPD, xeroderma pigmentosum complementation; Ski, superkiller; Swi, switching; Snf, sucrose non-fermenting; RIG, retinoic acid-inducible gene; RH, RNA helicase; NS, non-structural; NPH, nucleoside triphosphate phosphohydrolase. [1]

3.1.2 **Superfamily 2**

The superfamily 2 is the largest superfamily, and most eukaryotic RNA helicases belong to this superfamily. Based on its Walker B motif consensus sequence it is also termed DExH/D helicase family [16]. It consists of 10 families, five of which (DEAD-box, DEAH/RHA, superkiller (Ski) 2-like, retinoic acid-inducible gene (RIG)-I-like, and viral DExH proteins and the non-structural/nucleoside triphosphate phosphohydrolase (NS3/NPH)-II family) contain mainly RNA helicases and are therefore called “RNA helicase”-families [1, 27] [Figure 3]. For the remaining five families no RNA helicases have been identified.

3.1.3 **Superfamily 3**

In contrast to SF1 and SF2, SF3 contains only four conserved helicase domains including the Walker A and B motifs. Members of this superfamily were originally identified in RNA and DNA viruses and there are no SF3 helicases encoded in the cellular genomes [28-30].

3.2 **Function of RNA helicases**

RNA helicases are involved in almost all aspects of RNA metabolism such as transcription, translation initiation, ribosome biogenesis, nuclear mRNA export, RNA degradation and stabilization, and nuclear and mitochondrial RNA processing for example splicing [31] (Figure 4). Roles of RNA helicases have also been implicated in cell growth and differentiation [31]

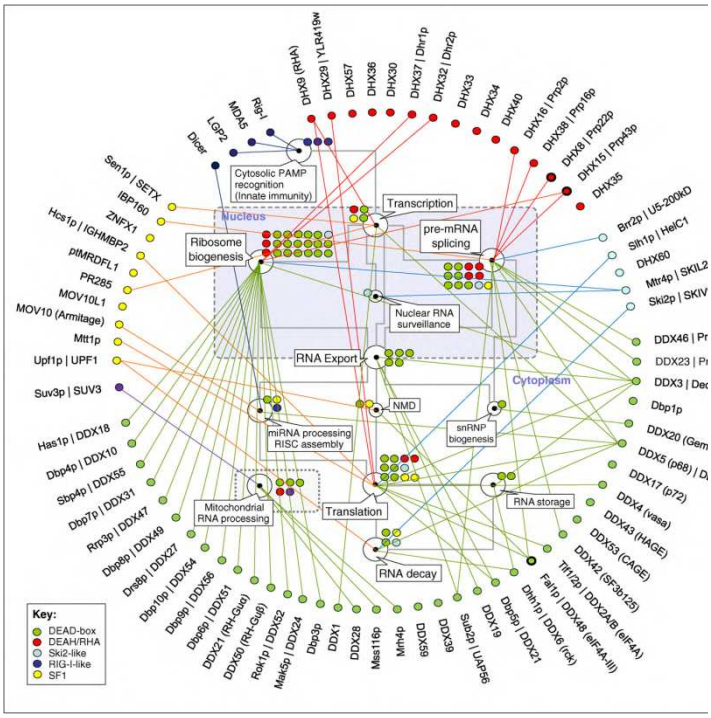


Figure 4: Cellular roles of eukaryotic RNA helicases. Selected, basic processes of eukaryotic RNA metabolism are represented by the white circles, as indicated by the callouts. The grey lines mark connections between processes. The colored circles represent the number of individual RNA helicases involved in a given process. RNA helicases (yeast and human orthologs) are grouped and color-coded according to their families (see legend at left lower corner). Connectors indicate involvement in one or more processes of RNA metabolism. Circles with bold lines emphasize the three RNA helicases (Prp22p, Prp43p, eIF4A-III) for which specific binding site information is available. [1]

RNA helicases have been originally defined on their capability to use the energy of NTP to unwind RNA duplexes. However, this unwinding activity has only been demonstrated so far *in vitro*. In the past years is has become clear that the function of RNA helicases is not limited to RNA strand separation but also include the displacement of proteins from RNA molecules, annealing of RNA strands, and stabilization of on-pathway folding intermediates.

In yeast, the majority of RNA helicases are involved in ribosome biogenesis and pre-mRNA splicing and many of them have homologs in eukaryotic cells [32]. This is not surprising since both processes require large ribonucleoproteins (RNPs).

3.2.1 **Translation**

The role of RNA helicases in mRNA translation (including translation initiation, elongation and termination) has been the subject of intense research in the last few years. Eukaryotic translation initiation of most mRNAs begins with the association of the 40S ribosomal subunit and the subsequent scanning for the initiation codon AUG [33]. This process is also controlled by the secondary structures of the mRNA in the 5'-untranslated region (UTR) since they must be unwind for optimal translation initiation [34]. The first RNA helicase described to play a role in translation initiation is the eIF4A [35]. It has been shown that eIF4A facilitates unwinding of 5'-UTR

secondary structures. Since then other RNA helicases have been described to play a role in initiation such as the RNA helicase DDX3 [36]. Recently, DHX29 has been described to play a role in translation of mRNAs harboring highly structured 5'-UTRs [37].

3.2.2 **Nuclear export**

Given that mRNAs are synthesized in the nucleus but translated within the cytoplasm they need to be translocated from the nucleus to the cytoplasm. Several RNA helicases are involved in this process. For instance, the human RNA helicase U2AF65-associated protein (UAP) 56 is a component of the transcription-export (TREX) complex and *knock down* of UAP56 results in accumulation of mRNAs in the nucleus [38, 39]. DEAD-box protein (Dbp) 5p, another RNA helicase, is suggested to play a role in releasing RNPs upon arrival in the cytoplasm and in coupling RNP export and translation [40]. The cap-binding protein eIF4E is involved in the nuclear export of a family of proliferation-related mRNAs that share a 50-nucleotide sequence element in their 3'-UTR [41].

3.2.3 **Degradation**

Messenger RNA decay is a highly regulated process playing a vital role in protein translation by way of regulation of the mRNA turnover rate. Furthermore, mRNAs harboring premature termination (nonsense) codons are degraded through a process termed nonsense-mediated mRNA decay (NMD) and fill as such an important function in protein translation surveillance. Many RNA helicases assist in these processes. For instance, the DExD/H-box helicase (DHH) 1 stimulates the mRNA decapping which is the initial step of RNA degradation [42]. The most prominent helicase involved in NMD is the SF 1 RNA helicase regulator of nonsense transcript (Rent) 1 [43]. Another helicase that plays a role in NMD is the RNA helicase DDX48, a member of the exon junction complex [44].

3.2.4 **Transcription**

A few RNA helicases have a function in gene transcription. It has been proposed that the cyclic adenosine monophosphate (cAMP) response element-binding protein (CBP) stimulates the expression of certain signal-dependent genes via its association with RNA polymerase II complexes [45]. The formation of the CBP-RNA polymerase II complex requires the RNA helicase A, and mutation of its helicase function reduces

cAMP response element binding (CREB)-dependent transcription [46]. P68, one of the first validated RNA helicases , acts as a transcriptional coactivator for the nuclear receptor estrogen receptor (ER) α [47]. Although the exact mechanism concerning the involvement of RNA helicases in transcription is not known, it is thought that most of them operate mainly as adaptors or bridges between factors in the transcriptional machinery [48].

3.2.5 **Pre-mRNA splicing**

One step in the correct formation of mRNA is the removal of introns from the mRNA precursors [49]. It is a multistep process initiated by the formation of a large RNA-protein complex, known as the spliceosome on the mRNA precursor [50, 51]. Many helicases play a fundamental role in this process. For example, p68 has been found to be part of an *in vitro*-reconstituted spliceosome, and depletion of p68 abolished the formation of the spliceosome [52, 53]. Other examples are the RNA helicases pre-RNA processing protein (Prp) 22p and Prp16p that use the energy of NTP hydrolysis to resolve RNA secondary structures within the spliceosome [54, 55].

3.2.6 **Ribosomal biogenesis**

Ribosomal biogenesis is a highly complex multistep process that leads to the formation of the ribosomal subunits. It involves over seventy ribosomal proteins, four ribosomal RNA (rRNA) molecules and several transacting factors. Most of the known RNA helicases are involved in these processes. For examples the RNA helicases Dbp6p, Dbp7p and suppressor of polyadenylated binding protein 1 (Spb) 4p are required for the 60S ribosomal subunit assembly in Saccharomyces cerevisiae [56-58]. Factor four A-like (Fal) 1p, another RNA helicase, leads to decreased 18S rRNA levels leading to an overall deficit in 40S ribosomal subunits [59].

3.3 **RNA helicase HELZ**

3.3.1 **Introduction**

Based on sequence comparison analysis, HELZ (**HEL**icase with **Z**inc finger motif) was assigned to the DNA2/nuclear accommodation of mitochondria 7 (DNA2/*NAM7)* helicase family of the superfamily 1 of RNA helicases. Additional members of this family include, among others, moloney leukemia virus (Mov) 10, Zinc finger (ZNF) X1 and Upf1. HELZ is alternatively called KIAA0054 or DRHC (**D**own-**R**egulated in

Human **C**ancer) [60, 61]. The HELZ gene is located on chromosomal band 17q25.1. The HELZ protein consists of 1942 amino acids, thus yielding a molecular weight of approximately 220 000 Daltons (Da).

3.3.2 **Structure**

In addition to the classical RNA helicase motifs, HELZ harbors a CCCH-Zinc finger at its N-terminal end. Furthermore, two conserved LxxLAP motifs can be detected at its C-terminal end, and a sequence comparison analysis identified a polyadenylated binding protein (PABP) association motif (PAM) 2 motif within the HELZ protein [10].

3.3.3 **Function**

The function of HELZ is poorly understood. The first published report on HELZ dates back to 1994 when a full-length HELZ complementary (c)DNA termed KIAA0054 was isolated from a human immature myeloid cell line cDNA library [60]. The cDNA encoded for a 1942 amino acid long protein and from the structure it was inferred that it contains a Walker A motif. Also, Northern blot analysis revealed ubiquitous expression of HELZ [60]. In 1999, Wagner et al. found that HELZ contains several motifs characteristic of members of the RNA helicase SF 1, including a putative purine nucleotide-binding site [62]. The study also identified the mouse homolog of HELZ and found widespread spatial and temporal expression throughout murine embryonic development [62]. Already a year later a research team from the Robert Wood Johnson Medical School in New Jersey aimed to identify novel Upf1 homologues by performing sequence comparisons. The group determined HELZ to belong to the Upf1-like subclass of helicases and observed the protein to contain, besides the classical seven RNA helicase signature motifs, two additional motifs [20]. It has been suggested that members of this subgroup might play a role in RNA-dependent processes [20]. In 2003, a research group from Japan showed reduced HELZ mRNA expression in 28 out of 95 (29%) cell lines derived from a variety of human cancers [61]. Based on this finding HELZ was newly termed DRHC [61]. Furthermore, transfection of a DRHC expression vector inhibited growth of cancer cells in liquid medium or soft agar, suggesting a tumor suppressor function of HELZ [61]. Since then, two other reports have been published showing the methyltransferases SET and MYND domain (SMYD) 2 and SMYD3 to form a

ternary complex with RNA polymerase II and HELZ, suggesting a role for HELZ in gene transcription [63, 64].

Protein translation

3.4 Introduction

Translation is the last step in the conversion of DNA to protein where mRNA nucleotide sequence is decoded into a protein sequence. Translation is highly regulated since the smallest mistake could lead to a malfunctioning or non-functioning protein. Also, regulation at this level allows the rapid response to a change in the physiological condition of the cell, since the transcriptional machinery does not need to be turned on and the response can occur much more quickly at mRNA level.

3.5 Messenger RNA structure

The majority cellular mRNAs contain a 5‘-cap structure composed of a tri-phosphate-linked 7-methylguanosine (i.e. m^7G), and except for most histones, a polyadenylated (poly)(A) tail of about 50-300 nucleotides in length [65]. Specific proteins of the translational machinery recognize this structure and alteration in the structure may lead to inefficient translation of the specific mRNA [66]. For example, eIF4E is able to bind to the 5’-cap and depletion of eIF4E drastically reduces cap-dependent translation [67, 68]. Messenger RNAs may contain secondary structures (mainly hairpins) within their 5’-UTR and mRNA translation efficiency is also dependent on the stability and position of these secondary structures [69].

3.6 Process

Translation can be broken down into four stages: initiation, elongation, termination, and recycling. For most mRNAs, translation starts with the binding of the 43S preinitiation complex to the cap of the mRNA followed by the scanning in 3’ direction until a start codon (AUG) is recognized. In the next step, the 40S ribosome assembles with a initiator-methionyl-transfer-RNA (Met-$tRNA_i^{Met}$) in its peptidyl (P)-site at the start codon and joins with the 60S ribosome to create the translationally active 80S initiation complex. Once the initiation complex is assembled elongation begins. During elongation, the nucleotide codon of the mRNA is translated within the ribosome to a polypeptide chain. Once a stop codon is reached, elongation is terminated and the 80S ribosomal complex is dissociated, preparing the ribosomal subunits for a new round of translation.

3.6.1 **Initiation**

Translation initiation is multistep process involving the 40S and 60S ribosomal subunits and several eukaryotic translation initiation factors [70]. Initially, the 43S preinitiation complex (comprising a 40S subunit, the eIF2–GTP–Met-tRNA$^{Met}_{i}$ ternary complex and several eukaryotic initiation factors including eIF1, 1A, 2, 3, and 5) attaches to the 5‘-cap of the mRNA through interaction with the trimeric eI4F scaffold (comprising eIF4A, eIF4E and eIF4G), notably with the rate-limiting initiation factor eIF4E that directly binds to the cap [71]. Next, the 43S complex scans along the 5‘-UTR in 5‘-3‘direction until it reaches the initiation codon AUG [33]. eIF4A assists in this process since it functions as a RNA helicase unwinding secondary structures [72]. Translation initiation efficiency is further enhanced through the interaction of the eIF4F complex with the poly(A) binding protein (PABP) that results in the circularization of the mRNA [8]. How this process leads to enhanced mRNA translation is not yet fully understood. Because PABP interacts with the eukaryotic release factor (eRF) 3, it has been suggested that circularization of the mRNA ameliorates ribosomal subunit recycling for the translation initiation of the same mRNA [9]. Also, eIF4F affinity for the cap is enhanced through the circularization of the mRNA [73]. Another report suggests that 60S subunit joining is enhanced through the interaction of PABP with eIF4F [74]. After initiation, codon recognition, and 48S complex formation, eIF5 and eIF5B promote the hydrolysis of eIF2-bound guanosine tri-phosphate (GTP), the displacement of the eIFs and the joining of a 60S subunit to form the active translational complex 80S [75]. The different initiation steps are summarized in Figure 5.

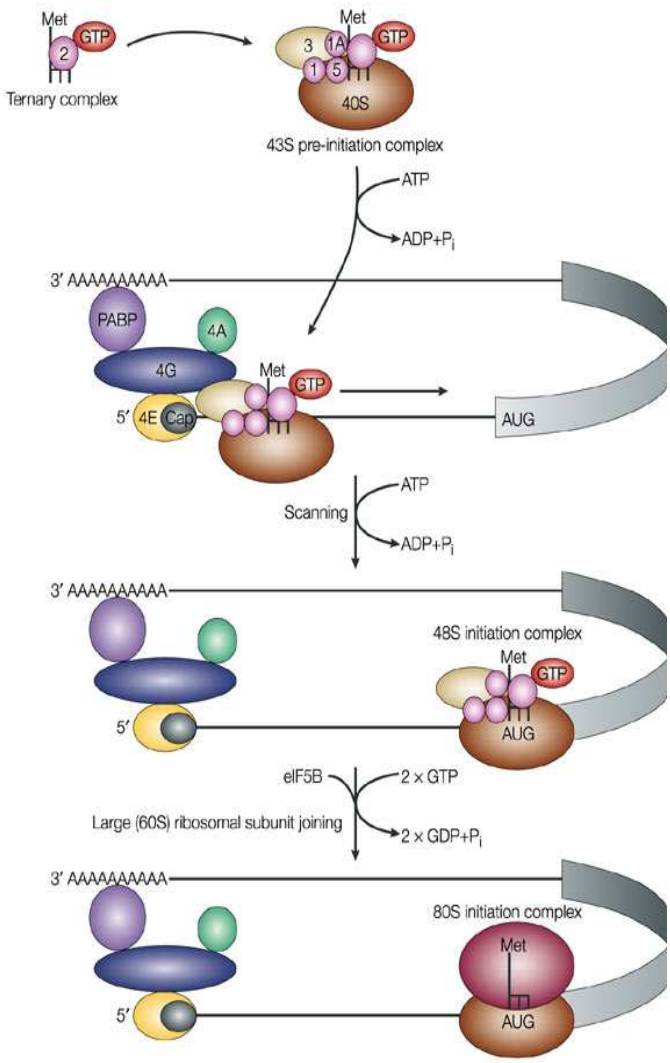


Figure 5: The methionine-loaded initiator tRNA (L-shaped symbol) binds to GTP-coupled eIF2, to yield the ternary complex. This complex then binds to the small (40S) ribosomal subunit, eIF3 and other initiation factors to form the 43S pre-initiation complex. The pre-initiation complex recognizes the mRNA by the binding of eIF3 to the eIF4G subunit of the cap-binding complex. In addition to eIF4G, the cap-binding complex contains eIF4E, which directly binds to the cap, and eIF4A, an RNA helicase that unwinds secondary structure during the subsequent step of scanning. eIF4G also contacts the poly(A)-binding protein (PABP) and this interaction is thought to circularize the mRNA. The 43S pre-initiation complex scans the mRNA in a 5' to 3' direction until it identifies the initiator codon AUG. Scanning is assisted by the factors eIF1 and eIF1A. Stable binding of the 43S pre-initiation complex to the AUG codon yields the 48S initiation complex. Subsequent joining of the large (60S) ribosomal subunit results in the formation of the 80S initiation complex. Both AUG recognition and joining of the large ribosomal subunit trigger GTP hydrolysis on eIF2 and eIF5B, respectively. Subsequently, the 80S complex is competent to catalyze the formation of the first peptide bond. P_i, inorganic phosphate. [2]

8.1.3 **Elongation**

Three positions are known to exist in the ribosome for the binding of tRNA: the acceptor (A)-site, the P-site and the exit (E)-site [76]. The three sites are named after the function of the tRNA at that specific position. At the A-site the correct aminoacyl-tRNA is accepted. At this site decoding of the mRNA takes place. In the P-site the tRNA with the polypeptide chain is located. The E-site is occupied by an empty tRNA prior to being released from the ribosome.

Elongation begins when the initiator tRNA occupies the P-site in the ribosome and the A-site is ready to accept an aminoacyl-tRNA. The addition of one amino acid to the peptide chain is a three step process. First a ternary complex composed of eukaryotic elongation factor (eEF) 1A, GTP and aminoacyl-t-RNA (carrying the anticodon corresponding to the codon adjacent to the initiation codon) is positioned at the A-site

of the ribosome. Second, the formation of the peptide bond is catalyzed through eEF1A-mediated hydrolysis of GTP to guanosine di-phosphate (GDP) [77, 78]. Third, once the bond is formed the mRNA is shifted by one codon by the eEF2 translocase [79]. This shifts the tRNA with its attached peptide to the P-site and opens the A site for the arrival of a new aminoacyl-tRNA. The deacetylated empty t-RNA is shifted to the E-site. This process is repeated until a stop codon is recognized and translation is terminated.

3.6.2 **Termination**

Two classes of eukaryotic polypeptide release factors govern the translation termination: class-1 (eRF1) and class-2 (eRF3) [80, 81]. If a stop codon lies in the A-site it is recognized by eRF1, and as a result eRF3, a GTPase bound to eRF1, hydrolyses GTP to GDP and the peptidyl-tRNA ester bond is cleaved, which causes the release of the newly formed peptide [82].

3.6.3 **Recycling**

Once the protein is released from the ribosome, the mRNA-tRNA-ribosome complex needs to be disassembled. In prokaryotes this process was first described already in 1972 but in eukaryotes only recently a model for ribosomal recycling has been suggested [83, 84]. In eukaryotes, recycling starts with the binding of eIF3 promoting release of the large (60S) ribosomal subunit [84]. In this model the 40S-mRNA-tRNA complex needs to be disassembled and this is facilitated through both the action of eIF1 ejecting deacylated tRNA from the P-site of the ribosome, and the action of eIF3j releasing all small ribosomal subunits from the mRNA [84, 85].

3.7 **Cytoplasmic poly(A) binding proteins (PABPs)**

Poly(A) binding proteins (PABPs) are a family of proteins characterized by their ability to bind the poly(A) RNAs via their RNA-recognition motifs (RRM). PABPs are found in yeast, plants, and animals but not in prokaryotes. PABPs are divided into two groups: nuclear and cytoplasmic. The human cytoplasmic PABP family consists of four members: PABP1, testis PABP (tPABP), inducible PABP (iPABP), and PABP5. Generally, PABPs consist of four non-identical RRMs connected to a carboxyl-terminal domain by a linker region that is rich in prolines and methionine residues [86]. In Figure 6 the overall structure of the different members of the PABP family is depicted.

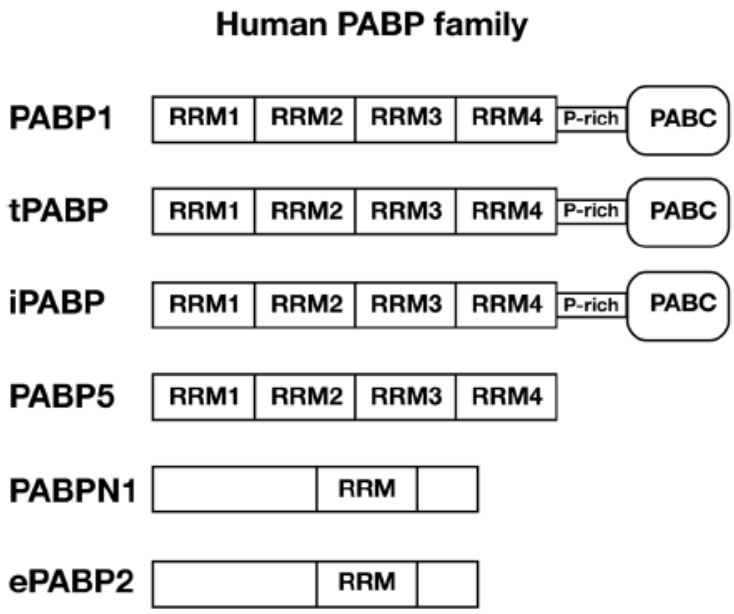


Figure 6: General structure of human poly(A)-binding protein (PABP) family members. P = proline. [87]

RRM1 und RRM2 bind poly(A) residues with high affinity, which leads to a globular structure that enables the simultaneous binding with other proteins [88]. PABPC1 also bind poly uracil and poly guanine although with lower affinity and does not bind poly cytosine [89]. At least 12 nucleotides are required *in vitro* for high affinity binding of RNA [90]. PABPs also interact with other proteins through its PABC domain. For example, the PABC domain mediates interaction with proteins that contain the PAM2 motif [91]. This motif has the consensus xxΦx(P/V)xAxxFxP, where Φ is a hydrophobic residue [92]. In Table 1 a number of known PAM2 motif-containing PABP interactors are shown.

Name	Position of first amino acid	Amino acid sequence
Paip1	123	SK**LS**VNAP**EF**Y**P**SGYSS
Paip2	109	SN**LN**PN**A**K**EF**V**P**GVKYG
Ataxin-2	912	ST**LN**PN**A**K**EF**N**P**RSFSQ
eRF3	50	RQ**LN**VN**A**K**PF**V**P**NVHAA
eRF3	59	FV**PN**VH**A**A**EF**V**P**SFLRG
Tob1	265	SA**LS**PN**A**K**EFIF**PNMQG
PAN3	84	KT**PN**PT**A**S**EFIP**KGGST
NF-X1	11	FK**FN**TD**A**A**EFIP**QEKKN

Table 1: PAM2 sequence of several known PABP interactors highlighting the most conserved amino acids is shown. Paip, PABP-interacting protein; Tob, transducer of Erb; PAN, poly(A)-specific nuclease; NF, nuclear factor. (adapted from [92])

3.7.1 **PABP1**

Alternative names are PAP1, PAB1, PAB, or PABC1. Its molecular weight is 70 000 Da. It is the most extensively studied member of the family. It is predominantly cytoplasmic, but can also shuttle to the nucleus [93, 94]. PABP1 functions synergistically with the eIF4F initiation complex to promote translation initiation [95]. It has been proposed that PABP also plays a role in ribosomal recycling through its interaction with the eRF3 [9]. Furthermore, PABP1 may also be important in mRNA decay by influencing the deadenylation dependent decapping pathway and exosome-mediated 3'-5' degradation [96]. A role for PABP in NMD and translation termination has also been proposed [97]. Just recently, PABP was found to interact with the C-terminus of GW182, a core component of the micro RNA-loaded RNA-induced silencing complex (miRISC), allowing docking of the deadenylase complex [98].

3.7.2 **Testis PABP**

Alternative name is PABPC3. Its mRNA is abundantly expressed in testis and can be detected in specific types of male germ cells suggesting a function in spermatogenesis [99]. It is homologous to PABP1 keeping the same overall structure with reduced affinity of its RRMs to RNA [100]. The homology to PABP1 suggests a role of tPABP in mRNA translation and stability but such a function has not yet been proven experimentally.

3.7.3 **Inducible PABP**

Alternative names are PABPC4 or activated-platelet protein (APP) 1. Similar to PABP1, iPABP is ubiquitously expressed in all tissues but at much higher levels in heart and skeletal muscle tissue than PABP [101]. The nomenclature refers to the proteins low level expression in resting T cells and platelets and rapid upregulation following T cell or platelet activation [101], [102]. Even though iPABP is closely related to PABP1 considering overall structure, subcellular localization, and tissue expression pattern no function for iPABP in translation or stability has been described yet.

3.7.4 **PABP5**

Alternative name is PABPC5. It is expressed in different kind of tissues such as adult brain, heart, liver, muscle, kidney and ovary, as well as fetal brain [103]. PABP5 lacks

the PABC domain and the linker region. No function has yet been attributed to PABP5.

Hypoxia

3.8 Introduction

In aerobic metabolism, energy that is stored in the form of ATP is mainly produced through a process termed oxidative phosphorylation. In this process, electrons are transferred through consecutive cytochromes to produce a proton gradient that is used to produce ATP. The final acceptor of the transferred electrons is oxygen. A constant oxygen supply is crucial for the maintenance of an adequate energy metabolism since incomplete transfer of electrons onto oxygen may lead to the production of harmful reactive oxygen species (ROS). Oxygen is distributed through the circulatory system via hemoglobin in the erythrocytes [104, 105]. To ensure sufficient oxygen transport to body tissues under low oxygen conditions such as at high altitude the body developed sophisticated physiological and molecular adaptation processes. In 1860, Viault observed an increase in red blood cell count at hight altitude [106]. In 1953, Allan Erslev discovered a humoral factor responsible for the increase in red blood cell production known today as erythropoietin (EPO) [107]. A major breakthrough in understanding the molecular mechanism behind Epo production came in 1991 when Wang and Semenza discovered the hypoxia inducible factor (HIF) to be responsible for the induced expression of the Epo gene during hypoxia through binding to a specific hypoxia-response element (HRE) in the 3' flanking sequence of the EPO gene [108]. HIF was originally found to be involved in regulation of EPO gene expression under hypoxic condition. However, since 1992 dozens of HIF-target genes were discovered many of them being involved in facilitating oxygen delivery and cellular adaptation to hypoxia by inducing multiple biological processes involved in oxygen-homeostasis, glucose-energy-metabolism, angiogenesis, metal transport and growth [109].

3.9 Hypoxia-inducible factor (HIF)

3.9.1 Expression and Composition

HIF is a heterodimeric protein complex consisting of an O_2-regulated constitutively expressed HIF-α subunit and an oxygen insensitive constitutively expressed HIF-β subunit. HIF-β is also known as aryl hydrocarbon receptor nuclear translocator

(ARNT), due to the observation that it dimerizes with the aryl hydrocarbon receptor (AhR) upon activation through binding of aryl hydrocarbons such as dioxin [110]. Both α and β subunits are members of the basic helix-loop-helix Per/Arnt/Sim (bHLH-PAS) transcription factor family [111]. For mammals, three isoforms of HIF-α are known: HIF-1α, HIF-2α (also known as endothelial PAS domain protein (EPAS) 1 or HIF-1α like factor (HLF)) and HIF-3α. In addition, three ARNT variants exist: ARNT, ARNT2 and ARNT3 [112, 113]. HIF-1α does not strictly associate with HIF-1β but can also dimerize with ARNT2 and ARNT3 [112, 113]. HIF-1α is ubiquitously expressed in all tissues and organs whereas HIF-2α and HIF-3α are more tissue-specific. HIF-2α has originally been described to be expressed primarily within vascular endothelial cells but later found to occur in fibroblast and epithelial cell lines as well [114, 115]. HIF-3α is primarily expressed within the kidney [116]. HIF-α protein consists of an N-terminal bHLH domain, that is necessary for DNA binding, and a PAS domain that is responsible for dimerization with ARNT [117]. Additionally, two transactivation domains (TADs) are found within the HIF-1α and -2α protein. Of those, the C-terminal domain is responsible for the recruitment of transcriptional co-activators crucial for target gene expression [117]. Furthermore, HIF-α contains two domains that explicitly trigger the normoxic degradation of the protein termed oxygen-dependent degradation (ODD) domain [118]. A nuclear localization signal (NLS) can also be found in the protein [119, 120]. In Figure 7 the overall structures of HIF-α and –β is summarized.

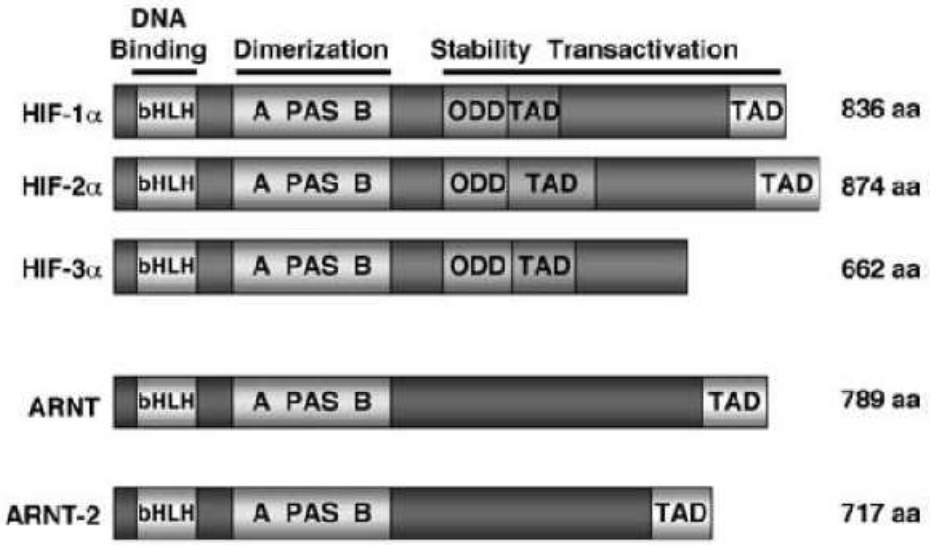


Figure 7: Schematic representation of HIF family member protein domains. [121]

3.9.2 **Regulation**

HIF is a central transcription factor that can control dozens of target genes. Clearly, HIF regulation is of crucial importance for cellular adaptations to periods of oxygen deprivation. Many cancers that are associated with the von Hippel-Lindau syndrome such as renal cell carcinoma are highly vascularized and expressed high levels of angiongenic factors such as vascular endothelial growth factor (VEGF) [122, 123]. This phenotype is also due to the fact that HIF is constantly active in these cancers since the HIF-α subunit remains stable due to the mal-functioning of the VHL protein, a substrate recognition component of an E3 ubiquitin ligase complex [124]. To ensure that HIF is not active under normal physiological conditions but rapidly activated under low oxygen conditions the body developed several levels of regulation most importantly post translational but also at the transcriptional level.

3.9.2.1 *Oxygen-dependent regulation*

3.9.2.1.1 Stability

HIF-α and HIF-β transcripts and proteins are, in cultured cells at least, continuously synthesized independently of oxygen but only HIF-α proteins undergo oxygen-regulated degradation [3]. Under normoxic conditions, two prolines (HIF-1α: 402 and 564) within the ODD domain of the human HIF-α protein are hydroxylated by specific HIF prolyl-4-hydroxylases (PHDs) which enables the binding of pVHL [3]. HIF-α is ubiquitinated and subsequently targeted to the 26S proteasome for degradation [4, 5]. Under hypoxic conditions, the prolyl hydroxylation reaction is inhibited, which impedes the interaction of pVHL with HIF-α and results in HIF-α accumulation in the nucleus and dimerization with HIF-β [119, 125-127].

3.9.2.1.2 Transactivation activity

In addition to prolyl hydroxylation, HIF-α is hydroxylated in oxygenated cells at a specific asparagine residue (HIF-1α: Asn803) by another hydroxylase termed factor inhibiting HIF (FIH) [128, 129]. The nature of this hydroxylation is not to cause HIF-a degradation but rather to block the interaction of HIF with the coactivators p300 and CBP [130]. Like PHDs, FIH is inhibited under hypoxia due to limited molecular oxygen leading to an active transactivation site. In Figure 8 a schematic overview on the oxygen-dependent regulation of HIF is given.

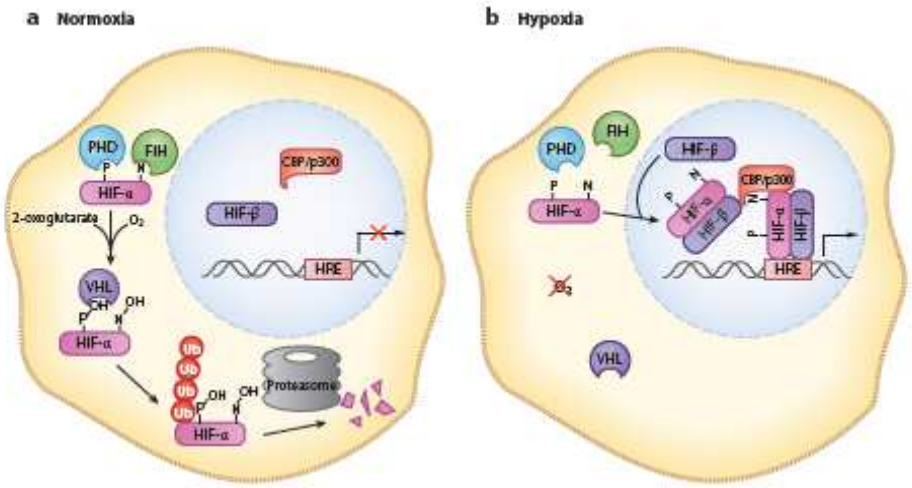


Figure 8: Oxygen-dependent regulation of hypoxia-inducible factor (HIF). (a) Under normoxic conditions, PHDs constitutively hydroxylate HIF-α on specific prolyl residues, and FIH constitutively hydroxylates HIF-α on a specific asparaginyl residue. Asparaginyl hydroxylation blocks the interaction between the transcriptional activation domain of HIF-α and the transcriptional co activator CBP/p300. Prolyl hydroxylation allows recognition by the VHL, a component of an E3 ubiquitin ligase complex that targets HIF-α for degradation by the proteasome. (b) Under hypoxic conditions, inhibition of prolyl hydroxylation promotes stabilization of HIF-α, which allows dimerization with HIF-β. Inhibition of asparaginyl hydroxylation unmasks the transcriptional activity of HIF-α, which allows binding of CBP/p300. [131]

3.9.2.2 *Oxygen-independent regulation*

In addition to the oxygen-dependent posttranslational regulation of HIF-α, a number of other mechanisms contribute to the downregulation of HIF activity or HIF protein levels. For example, the CBP/p300-interacting transactivators with glutamic acid (E) and aspartic acid (D)-rich tail (CITED) 2 inhibits HIF transactivation activity by blocking the HIF-1α–p300 interaction [132]. Another negative regulator of HIF is the acetyltransferase ARD1, a HIF-1 interactor that acetylates HIF at lysine 532 in the HIF-1α ODD domain. ARD1 enhances the HIF-1α-VHL interaction and leads to an increase in VHL mediated ubiquitination and subsequent proteasomal degradation of HIF [133].

Besides post-translation events, HIF-α transcription or translation can be considered as another level of regulation. For example, it was observed that stimulation of cells with a variety of growth factors or cytokines induces the expression of HIF-1α under normoxic condition which, in turn, resulted in the expression of several HIF-target genes [134-136]. These activations involve mainly the mammalian target of rapamycin (mTOR) and mitogen-activated protein kinase (MAPK) signaling pathways. For instance, overexpression of the human epidermal growth factor (Her) 2 in mouse 3T3 cells or hergulin stimulation of human MCF-7 cells resulted in increased HIF-1α protein translation and VEGF mRNA expression [137]. This increase was dependent upon activity of PI3K, AKT, and FRAPS [137]. A report

from 1999 shows that Interleukin (IL) 1α and Tumor necrosis factor (TNF) α can increase HIF expression and normoxic DNA-binding activity [138, 139]. Furthermore, it has been demonstrated that the promyelocytic leukaemia (PML) tumor suppressor inhibits HIF-1α translation through repression of the mammalian target of ramamycin (mTOR) function [140]. The MAPK pathway may also be involved in regulation of HIF activity. For example, p42/p44 MAPK phosphorylates HIF-1α *in vivo* promoting the transcriptional activity of HIF-1α [141]. Also, hypoxia-activated GTPase Rac1 induces p38 MAPK activity, leading to HIF-1α phosphorylation and increased TAD function [142]. Interestingly, also a natural antisense transcript that is complementary to the 3′-UTR of HIF-1α mRNA exists that regulates the expression of HIF-1α on the mRNA level and is found to be overexpressed in nonpapillary kidney cancers [143]. The inhibitory PAS domain protein (IPAS), an alternative splice variant of HIF-3α, acts as a dominant negative transcription factor that represses the HIF-α activity [144].

.

3.10 **The HIF prolyl-4-hydroxylases**

3.10.1 **Discovery**

In 2001, two groups simultaneously discovered that oxygen-dependent hydroxylation of two prolines (402 and 564) in the ODD domain of human HIF-1α triggers the binding of pVHL to the protein [145, 146]. A few months later the enzymes catalyzing the hydroxylation reaction were identified [6, 147]. Epstein and colleagues revealed egg-laying abnormal (EGL) 9 to be the HIF prolyl hydroxylase in *Caenorhabditis elegans* and found three PHD genes that are homologous to EGL-9 in mouse and human. Originally termed PHD1, PHD2 and PHD3, other names have been used such as HIF prolyl hydroxylase (HPH) 3, HPH2, and HPH1 [147], or EGL nine homologue (EGLN) 2, EGLN1, and EGLN3 [148].

3.10.2 **Characterization**

3.10.2.1 *PHD1*

The PHD1 gene is located on chromosome 19q13.2 and encodes a protein of 407 amino acids giving it the size of 45 000 Da. Its mRNA is ubiquitously expressed within all tissues but especially abundant in testis [149]. Experiments using green fluorescence protein (GFP)-tagged PHD1 protein revealed subcellular localization

within the nucleus [150]. Another study using monoclonal PHD1 antibodies to target the endogenous protein showed predominant cytoplasmic staining for PHD1 [151]. Homozygous *knock out* of PHD1 facilitated HIF-1α-mediated cardio-protection in ischemia/reperfused (I/R) myocardium and protected against the development of colitits through reduced epithelial cell apoptosis and increased barrier function [152-154].

3.10.2.2 *PHD2*

The PHD2 gene is located on chromosome 1q42-43 and encodes a protein of 426 amino acids giving it the size of 46 000 Da. Its mRNA is abundantly expressed in all tissues [149]. In normal tissue PHD2 is mainly expressed within the cytoplasm but increased nuclear PHD2 expression can be observed in less differentiated phenotypes of cancer cells [151, 155]. It has been postulated that PHD2 is the main regulator of HIF-1α [7]. PHD2 *knock out* embryos die between E12.5 and E14.5. Silencing PHD2 in adult mice causes increased renal EPO production and polycythemia [156, 157].

3.10.2.3 *PHD3*

The PHD3 gene is located on chromosome 14q13.1 and encodes a protein of 239 amino acids giving it the size of 27 000 Da. Its mRNA is ubiquitously expressed within all tissues but especially abundant in heart and liver [149]. PHD3 is expressed in cytoplasm as well as in the nucleus [151]. PHD3 is also termed smooth muscle (SM) 20 since it was found to be upregulated in rat smooth muscle cells after growth-factor stimulation [158]. *Knock out* of PHD3 in mice show reduced neuronal apoptosis, abnormal sympathoadrenal development, and reduced blood pressure [159].

3.10.3 **Enzymatic activity**

3.10.3.1 *Overview*

The PHDs belong to the superfamily of iron- and 2-oxoglutarate-dependent dioxygenases. Of the diatomic O_2 substrate, PHDs use one oxygen atom to hydroxylate prolines and the second one to convert 2-oxoglutarate to CO_2 and succinate. Fe^{2+} and ascorbate are cofactors in the reaction.

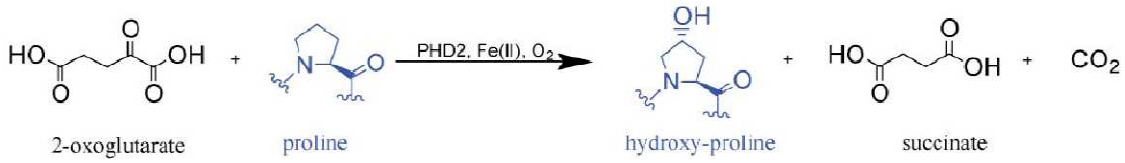


Figure 9: The reaction catalyzed by the PHDs . [160]

3.10.3.2 *Oxygen*

With the discovery of the PHD's in 2001 it was shown that the PHD enzymatic activity is highly sensitive to hypoxia [6]. In a study published in 2003 it was demonstrated that the K_m values for oxygen for all three PHDs ranged between 230-250 μM [161]. As this low affinity corresponded to a concentration slightly above the level of dissolved oxygen in air, it suggested PHDs to function as *bona fide* HIF oxygen sensors [161]. Interestingly, PHD2 and PHD3 are themselves HIF-1 target genes. In 2006, Stiehl and colleagues suggested an autoregulatory mechanism by which increased PHD2 and PHD3 protein abundances compensate for the reduced oxygen availability in recurring or prolonged hypoxia to reset HIF sensory thresholds [162].

3.10.3.3 *Iron and ascorbate*

PHDs are non-heme Fe-binding dioxygenases. Iron chelators, such as desferrioxamine, can stabilize the HIF-1α/-2α proteins in normoxia and trigger the induction of the HIF target gene presumably via the inhibition of PHD activity [163, 164]. It has already been known since the 1950s that cobalt chloride could be used for the treatment of sickle cell anemia [165]. The discovery of the PHDs shed light on the mechanism by which cobalt chloride is beneficial for the treatment of anemia: Cobalt and other transition metal ions such as nickel and manganese might compete with iron for the active site in the HIF sensor, thereby mimicking hypoxia-inducing HIF target gene expression [166]. Ascorbate plays an important function in the hydroxylation activity of the PHDs since it protects the enyzme's amino acid residues and/or active center iron from being oxidized [167]. In line with that finding, it has been shown that ascorbate is promoting PHD activity in cancer cells [168]. In another publication Nytko and colleagues showed that ascorbate is essential for PHD function *in vitro* [169].

3.10.3.4 *Citric acid cycle intermediates*

2-oxoglutarate is a key intermediate in the citric acid cycle and functions as cosubstrate for PHD-mediated hydroxylation. Fumarate hydratase (FH) and Succinate dehydrogenase (SDH) are enzymes of the citric acid cycle that catalyze the reduction of fumarate to L-malate and succinate to fumarate, respectively. I has been shown that germline mutations in FH can cause leiomyomas and renal cancer, whereas mutations in SDH may cause paragangliomas and phaeochromocytomas [170, 171]. Accumulation of these citric acid cycle intermediates is accompanied by the accumulation of the HIF-1α protein [172]. It has been suggested that loss-of-function of these enzymes causes the activation of the HIF-pathway by inhibiting PHDs since accumulation notably of succinate (i.e. in cases of SDH loss-of-function mutations) causes product inhibition of the PHDs. [173, 174].

3.10.4 **Substrate specificity**

All three PHDs hydroxylate the proline residue that is found within an LxxLAP motif (where x is any amino acid and P is the hydroxylacceptor proline) *in vitro* [175]. This motif is conserved in both hydroxylation sites and between different HIF isoforms. Surprisingly, mutation of any of the amino acids within the motifs revealed that only the proline is critical for the hydroxylation reaction [176]. Interestingly, it was demonstrated that a leucine, 10 residues C-terminal of both hydroxyprolines, is essential for VHL recognition [177]. In the last few years other proteins that are hydroxylated in the context of a LxxLAP motif have been described. For example, Kuznetsova and colleagues showed that RNA polymerase subunit B1 (Rpb1), the major subunit of the RNA polymerase II, is hydroxylated by PHDs (mainly PHD1) and targeted by pVHL [178]. A study performed in 2005 revealed 5000 different proteins containing one ore more LxxLAP motifs [179]. They found that the erythroid-specific aminolevulinate synthase (ALAS) 2 contains an LxxLAP motif and it has been shown that ALAS2 is hypoxically upregulated [180]. In 2006, Cummins and colleagues demonstrated that hypoxia increases both the expression and activity of IκB kinase (IKK) β, and site-directed mutagenesis of the proline residue (P191A) of the putative IKKβ hydroxylation site resulted in a loss of hypoxic inducibility [181]. However, evidence for hydroxylation of the proline residue is still missing. The iron regulatory protein (IRP) 2 has been suggested to be regulated by PHDs. For example, degradation of IRP2 is inhibited by hypoxia, desferrioxamine or DMOG

treatment [182, 183] – the latter two compounds known to mimic low oxygen levels by potently inactivating HIF prolyl and asparaginyl hydroxylases. Furthermore, IRP2 coimmunoprecipitates with VHL and promotes the ubiquitination and decay of transfected IRP2 [184]. However, no LxxLAP has been found in IRP2. The activating transcription factor (ATF) 4 was found to interact with PHD3 and ATF4 protein stability were increased by hypoxia or PHD inhibition. In addition, siRNA-mediated down-regulation of PHD3 resulted in increased ATF4 protein levels [185]. However, if ATF4 is a novel PHD3 hydroxylation substrate remains unknown.

3.10.5 **PHD2 interactors**

In the last few years a number of novel PHD interactors have been described. This includes novel PHD hydroxylation targets but also proteins that regulate PHD function through modulating enzymatic activity or stability. For example, it has been shown by mass spectrometry analysis that the RING finger E3 ubiquitin ligase seven in absentia homolog (SIAH) 2 interacts with PHDs with the following tight-weak relative ranking: PHD3 > PHD1 >> PHD2 [186]. The same work documented PHD3 stability to be regulated by SIAH2 via the targeting of PHD3 for proteasomeal degradation [186]. FKBP38, a peptidyl-prolyl cis/trans isomerase (PPIase) interacts specifically with PHD2 where it functions as adaptor in mediating the interaction of PHD2 with the proteasome to initiate the degradation of PHD2 [187, 188]. Other proteins have been described as putative novel PHD targets. For example, siRNA-mediated *knock-down* of PHD1 and PHD2 as well as treatment of cells with the PHD inhibitor DMOG resulted in NFkappaB induction [181]. Furthermore, overexpression of PHD1 decreased cytokine-stimulated NFκB reporter activity [181]. This finding suggested that IKKB might be a novel PHD hydroxylation target. Another protein reported to be hydroxylated in a PHD-dependent manner is Rpb1 [189]. Rpb1 can bind PHD1 and PHD2 and PHD1 (but not PHD2 and PHD3) was necessary for oxidative-stress-induced P1465 hydroxylation [178, 189].

Part 4: Manuscript I

The Putative RNA Helicase HELZ Promotes Cell Proliferation, Translation Initiation and Ribosomal Protein S6 Phosphorylation

Philippe A. Hasgall[1], David Hoogewijs[1], Marius B. Faza[2], Vikram G. Panse[2], Roland H. Wenger[1*], and Gieri Camenisch[1]

[1]Institute of Physiology and Zürich Center for Integrative Human Physiology ZIHP, University of Zürich UZH, CH-8057 Zürich, Switzerland
[2]Institute of Biochemistry, ETH Zürich, CH-8093 Zürich, Switzerland

[*]**Correspondence:** Roland H. Wenger, Institute of Physiology, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland. Tel: +41 (0)44 6355065; Fax: +41 (0)44 6356814; E-Mail: roland.wenger@access.uzh.ch

Running Title: HELZ activates translational initiation

Keywords: translation, RNA helicase, poly(A)-binding protein, prolyl-4-hydroxylation, mTOR signaling

Abstract

The hypoxia-inducible transcription factor (HIF) is a key component of the cellular adaptation mechanisms to hypoxic conditions. HIFα subunits are degraded by prolyl-4-hydroxylase domain (PHD) enzyme-dependent prolyl-4-hydroxylation of LxxLAP motifs that confer oxygen-dependent proteolytic degradation. Interestingly, only three non-HIFα proteins contain two conserved LxxLAP motifs, including the putative RNA helicase with a zinc finger domain HELZ. However, HELZ proteolytic regulation was found to be oxygen-independent, supporting the notion that a LxxLAP sequence motif alone is not sufficient for oxygen-dependent protein destruction. Since biochemical pathways involving RNA often require RNA helicases to modulate RNA structure and activity, we used luciferase reporter gene constructs and metabolic labeling to demonstrate that HELZ overexpression activates global protein translation whereas RNA-interference mediated HELZ suppression had the opposite effect. Although HELZ interacted with the poly(A)-binding protein (PABP) via its PAM2 motif, PABP was dispensable for HELZ function in protein translation. Importantly, downregulation of HELZ reduced translational initiation, resulting in the disassembly of polysomes, in a reduction of cell proliferation and hypophosphorylation of ribosomal protein S6.

Introduction

RNA helicases use ATP to modulate the structure of RNA, thereby altering the biologic activity of the RNA molecule or regulating access by other proteins. Virtually all biochemical processes involving RNA, including transcription, splicing, transport, translation, decay and ribosome biogenesis, employ helicases [1, 2]. The putative RNA helicase with a zinc finger domain HELZ belongs to the Upf1-like family of the superfamily I class of helicases [3, 4]. Members of this family have previously been implicated in mRNA processing [5, 6]. Tagged-HELZ has been shown to co-immunoprecipitate with co-transfected histone methyltransferases Smyd2 and Smyd3 and a functional role as adaptor molecule to RNA polymerase II has been suggested [7, 8]. However, the function of HELZ, especially in RNA metabolism, remained incompletely understood.

We identified two conserved LxxLAP motifs in the HELZ protein sequence which are known to be hydroxylated within the α-subunit of hypoxia-inducible transcription factors (HIFs). Transcriptional activation of oxygen-regulated genes by heterodimeric HIFs is a crucial step in the adaptation of mammalian cells to low oxygen [9, 10]. HIFα subunits are constitutively expressed but the protein stability is regulated by oxygen-dependent hydroxylation of specific prolyl residues located within the LxxLAP sequence by members of the prolyl-4-hydroxylase domain (PHD) enzyme family. Prolyl-4-hydroxylation is necessary for the interaction with the von Hippel-Lindau tumor suppressor protein (pVHL) that mediates HIFα degradation [11, 12, 13, 14, 15]. We report here that HELZ is oxygen-independently regulated, confirming that the sole presence of even two conserved LxxLAP motifs does not allow the prediction of oxygen-dependent regulation of protein stability.

Protein synthesis is regulated at various levels, but the limiting step is translational initiation. During this step, the 40S small ribosomal subunit is recruited to the mRNA 5’ end, scans towards a start codon and starts polypeptide synthesis by assembling the complete ribosome [16, 17]. Control of translation is often achieved by modulation of eukaryotic initiation factors (eIFs). The 5’ cap mRNA structure is bound by eIF4E, while eIF4G interacts with the poly(A)-binding protein (PABP) that associates with the 3’ poly(A) tail of eukaryotic mRNAs. eIF4A is a DEAD box RNA

helicase that resolves mRNA secondary structures and eIF4B harbors two RNA-binding domains interacting with mRNA and the 18S small ribosomal subunit [18]. Whereas translational initiation is suppressed by association of 4E-binding proteins to eIF4E, translation is activated following 40S ribosomal protein S6 kinase (S6K)-mediated phosphorylation of eIF4B that is then recruited to the initiation complex and enhances RNA helicase activity of eIF4A. Central in the regulation of the activity of these translation initiation factors is the mammalian target of rapamycin (mTOR) signal transduction pathway that integrates information on nutrients, energy, stress, hormones and mitogens with modulation of protein synthesis [19].

Here, we provide evidence that HELZ is involved in global protein translation. Mechanistically, HELZ downregulation resulted in S6 hypophosphorylation and dissociation of polysomes, suppressing cell proliferation.

Results

The putative RNA helicase HELZ contains two conserved LxxLAP motifs

A comprehensive *in silico* screen for proteins containing two LxxLAP motifs, like the HIFα subunits, revealed several candidate LxxLAP containing proteins in the human genome. To narrow down this list of proteins we further evaluated conservation across several species, which is generally considered suggestive for functionality of a sequence motif. Surprisingly, besides HIF-1α and HIF-2α only three additional such proteins were identified: HELZ, CCR4-NOT transcription complex (CNOT1) and faciogenital dysplasia protein (FGD1) (Supporting Table 1). Whereas CNOT1 is important for regulation of mRNA synthesis and decay [20], FGD1 encodes a guanine nucleotide exchange factor, activating the GTPase Cdc42 that is important for regulation of membrane trafficking [21]. However, none of these proteins have previously been reported to be oxygen-dependently regulated.

In our further studies, we concentrated on HELZ (accession number NP_055692.2), because coincidentally the same protein has been identified in a previously performed yeast two-hybrid screen for PHD2 interactors [22], suggesting that the two conserved LxxLAP motifs might be of functional relevance for interaction with the PHD oxygen sensors and probably oxygen-dependent regulation of HELZ protein stability and/or function. HELZ belongs to the superfamily I class of RNA helicases and contains a N-terminal C3H1-type zinc finger as well as typical helicase motifs including an ATP-binding domain (Walker A) important for helicase activity, a DEAA and a PAM2 motif, known to be required for interaction with the poly(A) binding protein (PABP) (Figure 1A). Both the N-terminal LxxLAP sequence that is part of the PAM2 motif, as well as the C-terminal LxxLAP motif are well conserved in mammals (Figure 1B upper and lower panel, respectively). The N-terminal LxxLAP motif is conserved to lower vertebrates like teleosts, whereas the C-terminal LxxLAP sequence is present only in placental mammals.

HELZ protein abundance is oxygen-independently regulated

To analyze whether the LxxLAP sequences of HELZ might have similar proteolysis regulating function as in HIFα subunits, we cultured human embryonic kidney

HEK293 cells for different time periods under normoxic or hypoxic conditions and investigated HELZ protein abundance by immunoblotting (Figure 2A). However, whereas HIF-1α protein levels were hypoxically induced as expected, HELZ protein expression was not significantly affected and variations are probably due to difficulties in detecting the predicted 219 kDa protein by immunoblotting. Also the pan-PHD inhibitor dimethyloxalylglycine (DMOG) did not affect HELZ protein levels (data not shown). Anti-HELZ antibody specificity was verified by RNA interference-mediated HELZ knock-down in HEK293 cells (stable clone 4-10) as well as by using the Hodgkin's lymphoma-derived cell line DEV that contains a 3-megabase homozygous deletion at 17q24.1-24.2, which includes, amongst others, HELZ [23]. The cell line L428 is also derived from an Hodgkin's lymphoma, but wild-type for HELZ, and served as positive control (Figure 2A).

HELZ mRNA levels were determined by RT-qPCR and although showing some variations were not significantly regulated under hypoxic conditions (Figure 2B, left panel). Hypoxic PHD2 mRNA induction served as positive control (Figure 2B, right panel). Organ-specific expression of HELZ *in vivo* has not been reported so far. In the mouse, HELZ mRNA levels varied between different tissues with highest levels in testis and brain and lowest in spleen and uterus (Figure 2C). Mice treated for 4 hours with an inspiratory gas mixture containing 0.1% carbon monoxide, resulting in acute tissue hypoxia, displayed no significant variations in HELZ mRNA levels, whereas other HIF target genes have been found to be strongly induced in these tissue samples [24, 25]. Finally, HELZ subcellular localization was analyzed by confocal immunofluorescence microscopy and remained mainly cytoplasmatic with enhanced perinuclear staining, independent of oxygenation and PHD function (Figure 2D, upper panel). On the other hand, HIF-1α was almost undetectable under normoxic conditions and accumulated in the nucleus under hypoxic and PHD inhibiting conditions (Figure 2D, lower panel).

It has been shown that the HELZ interactor SMYD3 changes subcellular localization during cell cycle progression and several RNA helicases have been reported to shuttle between nucleus and cytoplasm [8, 26, 27] Thus, we synchronized the cell cycle of the human hepatoma cell line HuH-7 and examined HELZ subcellular localization during cell cycle progression by indirect immunofluorescence (Supporting Figure S1). However, HELZ subcellular localization was not altered

during cell cycle progression, remaining mainly in the cytoplasm. In summary, these data suggest that the two LxxLAP motifs of HELZ might have either oxygen-independent functions, or oxygen-dependent functions that are not involved in proteolytic regulation like in the case of HIFα subunits, or no function at all. However, PHD-dependent modification of HELZ might still regulate HELZ function(s) other than protein abundance.

HELZ interacts with the poly(A)-binding protein via its PAM2 motif

The 3' poly(A) tail of eukaryotic mRNAs is bound by PABP that interacts with eIF4G, a subunit of the multiprotein initiation complex that binds to the 5' cap structure thereby regulating the initiation of translation. The PAM2 motif has originally been identified in the PABP interacting proteins Paip1 and Paip2 which regulate the activity of PABP [28]. The PAM2 motif is highly conserved in HELZ and overlaps with the N-terminal LxxLAP motif (Figure 1B). Therefore, we analyzed whether HELZ is able to interact with PABP by performing pull-down assays using HeLa cell lysates and GST-tagged HELZ fragments. As shown in Figure 3A, the PAM2 motif-containing GST-HELZ$^{1023\text{-}1199}$ fragment interacted with PABP. GST-Paip2$^{106\text{-}127}$ and GST alone served as positive and negative controls, respectively. Equal input was controlled by Coomassie staining (data not shown). PABP from hypoxic HeLa cell extracts also associated with recombinant GST-HELZ$^{1023\text{-}1199}$ (Figure 3B) suggesting that the PABP:HELZ interaction was proline hydroxylation independent. Vice-versa, GST-PABP$^{554\text{-}636}$ incubated with HEK293 cell lysates interacted with endogenous HELZ (Figure 3C).

The phenylalanine residue in the PAM2 motif of Paip1 and Paip2 is known to be critical for PABP binding [29]. Mutation of the corresponding phenylalanine in HELZ (F1107A) abrogated association with PABP, whereas mutation of the proline residue within the N-terminal LxxLAP sequence (P1105A) had no effect (Figure 3D). GST and GST-HELZ$^{1727\text{-}1942}$ served as negative controls. Furthermore, the HELZ PAM2 motif overlaps with the eIF4E-binding signature YxxxxLΦ (x designates any amino acid and Φ a hydrophobic residue) [30] and we therefore tested, whether HELZ can interact with eIF4E. Wild-type PAM2 motif-containing HELZ fragments also interacted with eIF4E, but probably indirectly via binding to PABP since the

interaction was abrogated when the phenylalanine residue critical for PABP binding was mutated (F1107A) (Supporting Figure S2).

HELZ is involved in global protein translation

The identification of HELZ as a novel PABP-interacting protein suggested a role of HELZ in protein translation. Therefore, we co-transfected HeLa cells with V5-tagged HELZ together with a SV40-driven renilla luciferase reporter gene construct. Exogenous overexpression of increasing amounts of HELZ induced relative luciferase activities in a dose-dependent manner (Figure 4A). To investigate whether enhanced luciferase activities were due to upregulation of mRNA levels, we performed RT-qPCR analysis. However, V5-HELZ expression did not influence renilla luciferase mRNA levels (Figure 4B). These effects were promoter-independent as the same regulation was observed with thymidine kinase (TK) and cytomegalovirus (CMV) driven reporter plasmids (data not shown). Vice versa, siRNA-mediated suppression of endogenous HELZ expression reduced luciferase activities (Figure 4C, upper panel). The HELZ siRNA oligonucleotides #9 and #11 efficiently down-regulated HELZ expression as verified by immunoblotting, whereas #12 was ineffective and used as control (Figure 4C, lower panel). Taken together, these data suggest that HELZ positively regulates reporter gene translation rather than transcription or mRNA stability.

To further investigate the role of HELZ on *in cellulo* protein translation, we performed [^{35}S]-methionine incorporation assays. Global translation was significantly induced by exogenous HELZ expression (Figure 5A, left panel), confirming the function of HELZ in protein translation. Surprisingly, mutation of the phenylalanine residue critical for PABP association within the PAM2 motif of HELZ did not influence HELZ-dependent stimulation of translation (Figure 5A, left panel). Expression of the indicated constructs was controlled by immunoblotting (Figure 5A, right panel).

To further elucidate the mechanism of HELZ function in translation we performed additional mutagenesis studies (schematically depicted in Figure 5B). Whereas ATP-binding and ATP hydrolysis motifs have previously been demonstrated to be crucial for eIF4A helicase function [31], mutation of the corresponding residues in HELZ (GNT, DQAA and NEAA) resulted in maintained stimulatory effects on protein translation (Figure 5B, left panel). Moreover, neither proline mutations in the

PAM2 motif nor in the C-terminal LxxLAP motif significantly influenced the positive effect of HELZ on translation (Figure 5B, left panel). Exogenous expression of V5-tagged HELZ constructs was controlled by immunobloting (Figure 5B, right panel). In addition, siRNA-mediated suppression of PABP did not influence the stimulatory effect of HELZ on global protein translation, supporting the notion that HELZ function is PABP independent (Figure 5C, left panel). Expression of the transfected vectors and PABP knock-down efficiencies were controlled by immunoblotting (Figure 5C, right panel).

HELZ promotes cell proliferation and is involved in ribosomal protein S6 phosphorylation

Regulation of protein translation impacts cell growth and proliferation. Consistent with a reduction in global protein translation, proliferation of HeLa cells was reduced by siRNA-mediated HELZ suppression (Fig. 6A). Cell viability, as assessed by Trypan blue staining, was not affected in siRNA-silenced cells (data not shown), suggesting that HELZ modulates cell proliferation rather than cell death.

Cellular translation requires a multitude of resources and the mammalian target of rapamycin (mTOR) signaling pathway functions as pivotal integrator [19]. Thus, we analyzed components of the mTOR pathway in HELZ-silenced cells and found a significant downregulation of phosphorylated ribosomal protein S6 (p-S6) compared to control cells (Fig. 6B). Total protein levels of S6 and S6 kinase (S6K), as well as AKT and PABP were not affected. Importantly, stable HELZ downregulation in the HEK293 clone 4-10 (previously described in Fig. 1A) resulted also in attenuation of S6 phosphorylation (Fig. 6C). Clone 4-6 showed a somewhat intermediate HELZ and p-S6 suppression and clone 4-14 served as control, demonstrating dose-dependent attenuation of S6 phosphorylation in an independent tissue culture model.

To determine at which step HELZ is involved in translational regulation, we analyzed polysome profiles from HELZ-silenced and control siRNA oligonucleotides transfected cells. HELZ downregulation resulted in dissociation of polysomes with a concomitant increase in the amount of 80S ribosomes and 60S subunits (Fig. 6D). Consistent with a mainly cytoplasmatic localization, these data suggest that HELZ functions in translational initiation.

Discussion

Although the specific prolyl residues within the HIFα subunits hydroxylated by PHDs share the sequence LxxLAP, *in vitro* studies on hydroxylase activity using substrate mutations have indicated that very few residues outside the hydroxylated proline itself are critical for hydroxylation [32, 33]. Fragments encompassing the entire HIFα oxygen-dependent degradation (ODD) domain are much better PHD substrates than short 20 amino acid model peptide substrates [34, 35], suggesting that the enzyme-substrate interaction requires multiple contacts. Indeed, crystal structure analysis suggested that the target proline is inserted into the hydroxylase active site while the rest of the unstructured ODD domain adopts an extended conformation around the enzyme [36, 37]. Nevertheless, also non-HIFα proteins have been proposed to be hydroxylated by PHDs within the context of an LxxLAP motif. PHD1 has been reported to hydroxylate Rpb1, the large subunit of the RNA polymerase II, as well as the IκB kinase-β (IKKβ), leading to hypoxic activation of NFκB [38, 39]. Our data indicate that conserved LxxLAP motifs can also be found within the PABP-interacting motif PAM2, but the presence of this motif alone cannot be taken as prerequisite for oxygen-dependent regulation. Furthermore, also the only two other proteins harboring two conserved LxxLAP motifs CNOT1 and FGD1, have not been described in the context of oxygen-dependent regulation.

Translational control is a crucial mechanism for a rapid response to physiological changes. Binding of PABP to the poly(A) tail of the mRNA is an important step in translation initiation, since it mediates the circularization of the mRNA through interaction of PABP with eIF4G, a member of the translation initiation complex [40]. Several other PABP binding partners, such as Paip1 and Paip2 have been described to associate through the PABP-interacting motif 2 (PAM2) [28, 41] and modulate PABP function [42, 43]. A bioinformatic survey has identified, among others, HELZ as PAM2 motif containing protein [44] and our data confirm that HELZ interacts with the PAM2 motif with PABP. Surprisingly, mutation of the phenylalanine residue critical for PABP association within the PAM2 motif of HELZ and siRNA-mediated suppression of PABPC1 did not influence HELZ-dependent stimulation of translation, suggesting that the HELZ function in protein translation is independent of PABP.

However, we cannot rule out that other cytoplasmic PABPs or inducible PABP (iPABP) might compensate for PABPC1. Interestingly, it has been shown that the PABC1 interactor Tob also associate with iPABP [45] and we can not exclude that HELZ promotes its effect indirectly. Furthermore, PABP-independent regulatory mechanisms of translation initiation are known and HELZ function might be specific for such mRNAs [46]. In addition, the stimulatory function of the putative RNA helicase HELZ on global protein translation was independent of the motifs derived from known RNA helicases, suggesting that either RNA helicase activity is not required for function of HELZ in translation, HELZ is not a RNA helicase or contains additional helicase motifs as previously suggested by Czaplinski *et al.* [5].

Ribosomal protein S6 is located within the 40S small ribosomal subunit and is essential for the translation of mRNAs encoding important components of the translation machinery, such as ribosomal proteins and elongation factors [47]. Phosphorylation of S6 is regulated by mTOR complex 1 (mTORC1) that activates S6 kinase (S6K) as well as compensatory pathways involving AKT or MAPK. Although hypophosphorylation of S6 by HELZ downregulation was independent of total S6K levels, we cannot exclude that HELZ modulated S6K activity directly or alternatively by reducing the activity or expression of a S6K-specific phosphatase. It has been suggested that Type 1 phosphatases can dephosphorylate ribosomal protein S6 and interestingly in this regard, the mammalian alpha4 phosphoprotein, a regulator of the protein phosphatase 2, has recently been described to interact with PABP [48]. Clearly, further experiments are needed to reveal the mechanism by which HELZ promotes S6 phosphorylation.

Human HELZ has alternatively been termed down-regulated in human cancers (DRHC). However, this was based on semi-quantitative RT-PCR analysis of HELZ expression in 95 tumor cell lines and the observation that exogenous HELZ expression inhibited proliferation and colony formation of hepatoma cells *in vitro* [49]. Contrary, the only functional HELZ data published so far suggest an adaptory role for HELZ linking the histone methyltransferase Smyd3 to RNA polymerase II, thereby transactivating oncogenes, homeobox genes and cell cycle regulators [8]. Smyd3 is up-regulated in colorectal and hepatocarcinoma cell lines and Smyd3 overexpression elevated proliferation and colony-formation capacity *in vitro*, suggesting that HELZ functions rather as tumor promoting factor [8]. Although we did not analyse the role of HELZ using tumor formation assays, our findings that

HELZ functions as a positive factor in translation support the tumor promoting hypothesis suggested by Hamamoto *et al.*

In summary, we provide evidence that the presence of even two conserved LxxLAP sequence motifs is not indicative of oxygen-dependent proteolytic regulation and describe to our knowledge for the first time a functional role of HELZ in initiation of translation and ribosomal protein S6 phosphorylation.

Materials and methods

Plasmids

Unless otherwise indicated, cloning work was carried out using Gateway technology (Invitrogen, Basel, Switzerland) as described previously [22]. The HELZ$^{1-1942}$ cDNA was obtained from Origene (LabForce, Nunningen, Switzerland) and cloned into pENTR4 (Invitrogen). pDONR-HELZ$^{1727-1942}$ originated from the yeast two-hybrid screen as described previously [22]. Point mutations were generated by site-directed mutagenesis of pENTR4-HELZ$^{1-1942}$ using the QuickChange site-directed mutagenesis kit following the manufacturer's instructions (Stratagene, Agilent Technologies, Basel, Switzerland). The HELZ fragment corresponding to amino acids 1023 to 1199 was amplified by PCR from pENTR4-HELZ$^{1-1942}$. The expression vectors pGEX-6P-1-Paip2$^{106-127}$ and pcDNA3.1-Paip1 were kindly provided by K. Gehring and N. Sonenberg (McGill University, Montreal, Canada), respectively.

Cell culture and transfections

Human MCF-7 breast carcinoma, human HeLa cervical carcinoma and human embryonic kidney HEK293 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Sigma, Buchs, Switzerland) as described previously [50], and human hepatoma HuH-7 cells were cultured in RPMI-1640 [51]. For long-term hypoxia, cells were grown in a gas-controlled glove box (Invivo$_2$ 400, Ruskinn Technologies, Leeds, UK). Transfections were performed using polyethylenimine (Polysciences, Warrington, PA, USA) as described previously [25] or Lipofectamine2000 (Invitrogen).

Normoxic and hypoxic mouse tissue samples

Exposure of mice to an inspiratory gas mixture containing 0.1% carbon monoxide for 4 hours resulted in a 50% functional anemia and has been described elsewhere [24].

Immunoblotting

Immunoblot analyses were performed as previously described [52]. Antibodies used were mouse monoclonal antibody (mAb) anti-HIF-1α (Transduction Laboratories,

BD Biosciences, Allschwil, Switzerland), mAb anti-HELZ (Abnova, LucernaChem, Luzern, Switzerland), mAb anti-β-actin (Sigma), rabbit polyclonal antibody (pAb) anti-SP1 (Santa Cruz Biotechnology, LabForce, Nunningen, Switzerland), pAb anti-PABP (Abcam, LucernaChem), pAb anti-AKT, anti-S6K, anti-S6 and anti-pS6 (Cell Signaling, BioConcept, Allschwil, Switzerland).

mRNA quantification

Total cellular RNA was extracted as described previously [22]. First-strand cDNA synthesis was performed with 1-5 μg total RNA using reverse transcriptase (RT) and mRNA levels were measured by real-time quantitative (q) PCR using internal calibration standards and a SybrGreen qPCR reagent kit (Sigma) in combination with the MX3000P light cycler (Stratagene). To verify RNA integrity and equal input levels, ribosomal protein L28 or S12 mRNA was determined, and the data were expressed as ratios relative to L28 or S12 mRNA levels.

Immunofluorescence microscopy

Indirect immunofluorescence microscopy was performed as described previously [22]. For synchronization experiments, cells were growth-arrested in the G_0/G_1 phase by incubation with 5 μg/ml aphidicolin (Sigma) for 36 hours. Immunofluorescence staining was performed at times 0, 4, 8 and 12 hours after the withdrawal of aphidicolin. Cell cycle stage was determined by staining cells with propidium iodide and analyzed by flow cytometry (BD Biosciences).

Protein expression and purification

GST and GST fusion proteins were expressed in *Escherichia coli* BL21-AI by induction with 0.025% arabinose for 4 hours and affinity purified using glutathione-sepharose beads (GSTrap FF; GE Healthcare, Dübendorf, Switzerland) according to the manufacturer's instructions.

Pull-down assays

HeLa cells were grown to 80% confluency and lysed in buffer A (20 mM Hepes/KOH (pH 7.4), 100 mM KCl, 0.1% NP-40, 0.5 mM EDTA, 10% (v/v) glycerol and protease inhibitors (complete EDTA-free, Roche Applied Science, Rotkreuz, Switzerland).

Recombinant GST or GST-HELZ fusion proteins (0.5 μg) were incubated with total HeLa cell extracts (2.5 mg) in the presence of glutathione-sepharose beads for 3 hours at 4°C. Beads were washed ten times in buffer A and bound proteins were eluted by incubation in SDS-PAGE loading buffer.

Transient transfection and luciferase assay

Cells were co-transfected with the indicated firefly luciferase reporter gene plasmids together with 40 ng pRLSV40 renilla luciferase reporter gene vector (Promega, Dübendorf, Switzerland) and luciferase activity was determined using the dual-luciferase reporter assay system according to the manufacturer's instructions (Promega).

RNA interference

Cells were transfected with either 100 nM siRNA (Invitrogen) or 50 nM siRNA (Dharmacon, Fisher Scientific, Wohlen, Switzerland) duplex oligonucleotides using Lipofectamine 2000 (Invitrogen). The following forward siRNA oligonucleotides (Invitrogen) were used:

Control siRNA, 5'-GCUCCGGAGAACUACCAGAGUAUUA-3';

HELZ siRNA#2, 5′-UUCAGCUGCAUGCAGCAAAGCACUG-3′;

HELZ siRNA#3, 5′-AUGAGGUGGUGUUUACCCAGGACUC-3′.

The following forward siRNA oligonucleotides (Dharmacon) were used:

HELZ siRNA#09, 5'-CGUGUAUAAAGUCGGGAUA-3';

HELZ siRNA#11, 5'-GCAGUUGAUCCUCGAAUUA-3';

HELZ siRNA#12, 5'-AAUCACAAGCAGCGAGGAA-3';

PABPC1 siRNA#5, 5'-CAUGUAAGGUGGUUUGUGA-3';

PABPC1 siRNA#8, 5'-UGGAUGAGAUGAACGGAAA-3'.

The ON-TARGETplus siCONTROL non-targeting pool from Dharmacon was used as control. Expression vectors encoding shRNA sequences targeting HELZ driven by the U6 promoter in a pLKO.1-puro plasmid were purchased from Sigma.

Metabolic labelling

After transfection of cells with siRNA oligonucleotides or plasmids, cells were incubated for 2 hours in methionine-depleted medium supplemented with 10% (v/v)

dialyzed FCS. Cells were pulse-labeled with 20 μCi of [^{35}S]-methionine for 1 hour. Finally, cells were washed twice in ice-cold PBS and lysed with buffer containing 10 mM Hepes/KOH (pH 8.0), 1.5 mM $MgCl_2$, 10 mM KCl and 0.5% NP-40 supplemented with EDTA-free protease inhibitors for 15 minutes. Lysates were centrifuged for 15 minutes and protein concentrations determined. Total protein (10 μg) was precipitated by 10% trichloroacetic acid, washed twice in ice-cold acetone and radioactivity was measured by scintillation counting.

Polysomal profiles analysis

HeLa cells were transfected with siRNA oligonucleotides and 48 hours later treated with 100 μg/ml cycloheximide for 15 min at 37°C. Cells were then washed with ice-cold PBS and lysed by addition of 300 μl lysis buffer containing 10 mM Hepes/KOH (pH 7.5), 10 mM $MgCl_2$, 50 mM KCl, 2 mM DTT, 0.5% NP-40 and 1% deoxycholate supplemented with EDTA-free protease inhibitors for 15 minutes. Lysates were centrifuged for 15 minutes at 12'000 x *g*. The supernatant was loaded onto a 7-50% sucrose density gradient containing 50 mM Tris-acetate (pH 7.4), 50 mM NH_4Cl, 12 mM $MgCl_2$ and 1 mM DTT. The gradient was centrifuged at 39'000 rpm for 165 minutes at 4°C (SW41 Beckman Coulter) and analyzed at 254 nm using a density gradient fractionator.

Statistical analysis

Where not otherwise indicated, results are presented as mean values ± standard error of the mean of at least n = 3 independent experiments. All statistical tests were performed and graphed using GraphPad Prism v4.0 software (*, $P < 0.05$; **, $P < 0.01$).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

We would like to thank P. Spielmann for excellent technical assistance, K. Gehring and N. Sonenberg for gifts of plasmids, I. J. Frew for gifts of antibodies and T. A. Gorr, D. M. Katschinski and D. P. Stiehl for helpful discussions.

Funding

This work was supported by the Swiss National Science Foundation (31003A_129962/1). D. Hoogewijs is a recipient of a Marie Curie Intra-European Fellowship from the European Commission (PIEF-GA-2009-236157-HYDRELZ). V. G. Panse is supported by the Swiss National Science Foundation, the ETH Zürich and the European Research Council (EURIBIO-260676).

Note

Supporting information for this article is available on the PLoS ONE website (http://www.plosone.org).

References

1. Luking A, Stahl U, Schmidt U (1998) The protein family of RNA helicases. Crit Rev Biochem Mol Biol 33: 259-296.
2. Jankowsky E, Fairman ME (2007) RNA helicases-one fold for many functions. Curr Opin Struct Biol 17: 316-324.
3. Singleton MR, Dillingham MS, Wigley DB (2007) Structure and mechanism of helicases and nucleic acid translocases. Annu Rev Biochem 76: 23-50.
4. Fairman-Williams ME, Guenther UP, Jankowsky E (2010) SF1 and SF2 helicases: family matters. Curr Opin Struct Biol 20: 313-324.
5. Czaplinski K, Majlesi N, Banerjee T, Peltz SW (2000) Mtt1 is a Upf1-like helicase that interacts with the translation termination factors and whose overexpression can modulate termination efficiency. RNA 6: 730-743.
6. Dalmay T, Horsefield R, Braunstein TH, Baulcombe DC (2001) SDE3 encodes an RNA helicase required for post-transcriptional gene silencing in Arabidopsis. EMBO J 20: 2069-2078.
7. Diehl F, Brown MA, van Amerongen MJ, Novoyatleva T, Wietelmann A, et al. (2010) Cardiac deletion of Smyd2 is dispensable for mouse heart development. PLoS One 5: e9748.
8. Hamamoto R, Furukawa Y, Morita M, Iimura Y, Silva FP, et al. (2004) SMYD3 encodes a histone methyltransferase involved in the proliferation of cancer cells. Nat Cell Biol 6: 731-740.
9. Wenger RH (2002) Cellular adaptation to hypoxia: O_2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O_2-regulated gene expression. FASEB J 16: 1151-1162.
10. Kaelin WG, Jr., Ratcliffe PJ (2008) Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. Mol Cell 30: 393-402.
11. Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, et al. (1999) The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature 399: 271-275.
12. Bruick RK, McKnight SL (2001) A conserved family of prolyl-4-hydroxylases that modify HIF. Science 294: 1337-1340.
13. Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, et al. (2001) *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell 107: 43-54.
14. Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, et al. (2001) Targeting of HIF-α to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. Science 292: 468-472.
15. Ivan M, Haberberger T, Gervasi DC, Michelson KS, Gunzler V, et al. (2002) Biochemical purification and pharmacological inhibition of a mammalian prolyl hydroxylase acting on hypoxia-inducible factor. Proc Natl Acad Sci USA 99: 13459-13464.
16. Pestova TV, Kolupaeva VG, Lomakin IB, Pilipenko EV, Shatsky IN, et al. (2001) Molecular mechanisms of translation initiation in eukaryotes. Proc Natl Acad Sci USA 98: 7029-7036.
17. Gebauer F, Hentze MW (2004) Molecular mechanisms of translational control. Nat Rev Mol Cell Biol 5: 827-835.

18. Gingras AC, Raught B, Sonenberg N (1999) eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. Annu Rev Biochem 68: 913-963.
19. Ma XM, Blenis J (2009) Molecular mechanisms of mTOR-mediated translational control. Nat Rev Mol Cell Biol 10: 307-318.
20. Collart MA, Timmers HT (2004) The eukaryotic Ccr4-not complex: a regulatory platform integrating mRNA metabolism with cellular signaling pathways? Prog Nucleic Acid Res Mol Biol 77: 289-322.
21. Estrada L, Caron E, Gorski JL (2001) Fgd1, the Cdc42 guanine nucleotide exchange factor responsible for faciogenital dysplasia, is localized to the subcortical actin cytoskeleton and Golgi membrane. Hum Mol Genet 10: 485-495.
22. Barth S, Nesper J, Hasgall PA, Wirthner R, Nytko KJ, et al. (2007) The peptidyl prolyl cis/trans isomerase FKBP38 determines hypoxia-inducible transcription factor prolyl-4-hydroxylase PHD2 protein stability. Mol Cell Biol 27: 3758-3768.
23. Atayar C, Kok K, Kluiver J, Bosga A, van den Berg E, et al. (2006) BCL6 alternative breakpoint region break and homozygous deletion of 17q24 in the nodular lymphocyte predominance type of Hodgkin's lymphoma-derived cell line DEV. Hum Pathol 37: 675-683.
24. Marti HH, Wenger RH, Rivas LA, Straumann U, Digicaylioglu M, et al. (1996) Erythropoietin gene expression in human, monkey and murine brain. Eur J Neurosci 8: 666-676.
25. Stiehl DP, Wirthner R, Koditz J, Spielmann P, Camenisch G, et al. (2006) Increased prolyl 4-hydroxylase domain proteins compensate for decreased oxygen levels. Evidence for an autoregulatory oxygen-sensing system. J Biol Chem 281: 23482-23491.
26. Smillie DA, Sommerville J (2002) RNA helicase p54 (DDX6) is a shuttling protein involved in nuclear assembly of stored mRNP particles. J Cell Sci 115: 395-407.
27. Wang H, Gao X, Huang Y, Yang J, Liu ZR (2009) P68 RNA helicase is a nucleocytoplasmic shuttling protein. Cell Res 19: 1388-1400.
28. Roy G, De Crescenzo G, Khaleghpour K, Kahvejian A, O'Connor-McCourt M, et al. (2002) Paip1 interacts with poly(A) binding protein through two independent binding motifs. Mol Cell Biol 22: 3769-3782.
29. Kozlov G, De Crescenzo G, Lim NS, Siddiqui N, Fantus D, et al. (2004) Structural basis of ligand recognition by PABC, a highly specific peptide-binding domain found in poly(A)-binding protein and a HECT ubiquitin ligase. EMBO J 23: 272-281.
30. Mader S, Lee H, Pause A, Sonenberg N (1995) The translation initiation factor eIF-4E binds to a common motif shared by the translation factor eIF-4 gamma and the translational repressors 4E-binding proteins. Mol Cell Biol 15: 4990-4997.
31. Pause A, Sonenberg N (1992) Mutational analysis of a DEAD box RNA helicase: the mammalian translation initiation factor eIF-4A. EMBO J 11: 2643-2654.
32. Huang J, Zhao Q, Mooney SM, Lee FS (2002) Sequence determinants in hypoxia-inducible factor-1alpha for hydroxylation by the prolyl hydroxylases PHD1, PHD2, and PHD3. J Biol Chem 277: 39792-39800.
33. Li D, Hirsila M, Koivunen P, Brenner MC, Xu L, et al. (2004) Many amino acid substitutions in a hypoxia-inducible transcription factor (HIF)-1alpha-like peptide cause only minor changes in its hydroxylation by the HIF prolyl 4-hydroxylases: substitution of 3,4-dehydroproline or azetidine-2-carboxylic acid for the proline

leads to a high rate of uncoupled 2-oxoglutarate decarboxylation. J Biol Chem 279: 55051-55059.
34. Koivunen P, Hirsila M, Kivirikko KI, Myllyharju J (2006) The length of peptide substrates has a marked effect on hydroxylation by the hypoxia-inducible factor prolyl 4-hydroxylases. J Biol Chem 281: 28712-28720.
35. Hirsila M, Koivunen P, Gunzler V, Kivirikko KI, Myllyharju J (2003) Characterization of the human prolyl 4-hydroxylases that modify the hypoxia-inducible factor. J Biol Chem 278: 30772-30780.
36. McDonough MA, Li V, Flashman E, Chowdhury R, Mohr C, et al. (2006) Cellular oxygen sensing: Crystal structure of hypoxia-inducible factor prolyl hydroxylase (PHD2). Proc Natl Acad Sci USA 103: 9814-9819.
37. Sanchez-Puig N, Veprintsev DB,Fersht AR (2005) Binding of natively unfolded HIF-1α ODD domain to p53. Mol Cell 17: 11-21.
38. Mikhaylova O, Ignacak ML, Barankiewicz TJ, Harbaugh SV, Yi Y, et al. (2008) The von Hippel-Lindau tumor suppressor protein and Egl-9-Type proline hydroxylases regulate the large subunit of RNA polymerase II in response to oxidative stress. Mol Cell Biol 28: 2701-2717.
39. Cummins EP, Berra E, Comerford KM, Ginouves A, Fitzgerald KT, et al. (2006) Prolyl hydroxylase-1 negatively regulates IkB kinase-β, giving insight into hypoxia-induced NFκB activity. Proc Natl Acad Sci USA 103: 18154-18159.
40. Mangus DA, Evans MC, Jacobson A (2003) Poly(A)-binding proteins: multifunctional scaffolds for the post-transcriptional control of gene expression. Genome Biol 4: 223.
41. Khaleghpour K, Kahvejian A, De Crescenzo G, Roy G, Svitkin YV, et al. (2001) Dual interactions of the translational repressor Paip2 with poly(A) binding protein. Mol Cell Biol 21: 5200-5213.
42. Craig AW, Haghighat A, Yu AT, Sonenberg N (1998) Interaction of polyadenylate-binding protein with the eIF4G homologue PAIP enhances translation. Nature 392: 520-523.
43. Khaleghpour K, Svitkin YV, Craig AW, DeMaria CT, Deo RC, et al. (2001) Translational repression by a novel partner of human poly(A) binding protein, Paip2. Mol Cell 7: 205-216.
44. Albrecht M, Lengauer T (2004) Survey on the PABC recognition motif PAM2. Biochem Biophys Res Commun 316: 129-138.
45. Okochi K, Suzuki T, Inoue J, Matsuda S, Yamamoto T (2005) Interaction of anti-proliferative protein Tob with poly(A)-binding protein and inducible poly(A)-binding protein: implication of Tob in translational control. Genes Cells 10: 151-163.
46. Sachs AB, Sarnow P, Hentze MW (1997) Starting at the beginning, middle, and end: translation initiation in eukaryotes. Cell 89: 831-838.
47. Dufner A, Thomas G (1999) Ribosomal S6 kinase signaling and the control of translation. Exp Cell Res 253: 100-109.
48. McDonald WJ, Sangster SM, Moffat LD, Henderson MJ, Too CK alpha4 phosphoprotein interacts with EDD E3 ubiquitin ligase and poly(A)-binding protein. J Cell Biochem 110: 1123-1129.
49. Nagai H, Yabe A, Mine N, Mikami I, Fujiwara H, et al. (2003) Down-regulation in human cancers of DRHC, a novel helicase-like gene from 17q25.1 that inhibits cell growth. Cancer Lett 193: 41-47.
50. Camenisch G, Tini M, Chilov D, Kvietikova I, Srinivas V, et al. (1999) General applicability of chicken egg yolk antibodies: the performance of IgY

immunoglobulins raised against the hypoxia-inducible factor 1α. FASEB J 13: 81-88.
51. Popowski K, Eloranta JJ, Saborowski M, Fried M, Meier PJ, et al. (2005) The human organic anion transporter 2 gene is transactivated by hepatocyte nuclear factor-4 alpha and suppressed by bile acids. Mol Pharmacol 67: 1629-1638.
52. Balamurugan K, Luu VD, Kaufmann MR, Hofmann VS, Boysen G, et al. (2009) Onconeuronal cerebellar degeneration-related antigen, Cdr2, is strongly expressed in papillary renal cell carcinoma and leads to attenuated hypoxic response. Oncogene 28: 3274-3285.
53. Sigrist CJ, Cerutti L, de Castro E, Langendijk-Genevaux PS, Bulliard V, et al. (2010) PROSITE, a protein domain database for functional characterization and annotation. Nucleic Acids Res 38: D161-166.
54. Blanchette M, Kent WJ, Riemer C, Elnitski L, Smit AF, et al. (2004) Aligning multiple genomic sequences with the threaded blockset aligner. Genome Res 14: 708-715.
55. Rhead B, Karolchik D, Kuhn RM, Hinrichs AS, Zweig AS, et al. (2010) The UCSC Genome Browser database: update 2010. Nucleic Acids Res 38: D613-619.

Figure legends

Figure 1

Human HELZ protein sequence analysis

(**A**) Schematic representation of the predicted human HELZ protein architecture. (**B**) Sequence alignment of the N-terminal LxxLAP motif that overlaps with the PAM2 motif (upper panel) and C-terminal LxxLAP motif (lower panel) of the indicated species. The LxxLAP motif is depicted in red and strictly conserved residues are shown as white letters on black background.

Figure 2

Oxygen-independent regulation of HELZ

(**A**) Immunoblot analysis of HEK293 cells cultured under normoxic or hypoxic conditions for the indicated time periods. The stable HELZ shRNA clone 4-10 and the Hodgkin's lymphoma-derived cell line DEV containing a homozygous deletion on chromosome 17 served as controls for reduced and absent HELZ protein levels, respectively. The Hodgkin's lymphoma derived cell line L428 served as positive control. (**B**) Transcript levels of HELZ were analyzed in HEK293 cells by RT-qPCR. Cells were exposed to hypoxia (0.2% O_2) for up to 48 hours and PHD2 served as hypoxia-inducible control. (**C**) Total RNA was extracted from tissue samples of various organs from mice exposed to inspiratory air (0% F_iCO) or 0.1% carbon monoxide (0.1% F_iCO) for 4 hours and HELZ transcript levels were quantified by RT-qPCR and normalized to ribosomal protein S12 mRNA levels. (**D**) MCF-7 cells were cultured under 20% O_2 or 0.2% O_2 for 18 hours, fixed, and HELZ as well as HIF-1α protein visualized by indirect immunofluorescence.

Figure 3

HELZ interacts with the poly(A)-binding protein (PABP)

GST pull-down using glutathione-sepharose beads was conducted by incubating crude HeLa cell lysates with recombinant GST, GST-Paip2$^{106\text{-}127}$ and GST-HELZ$^{1023\text{-}1199}$ (**A**), GST and the indicated GST-HELZ fragments (**D**), or crude HEK293 cell lysates with GST and GST-PABP$^{554\text{-}636}$ (**C**). Hypoxic HeLa cell lysates were incubated with

GST or GST-HELZ$^{1023-1199}$ (**B**). Eluates were subjected to SDS-PAGE and immunoblotting using the indicated antibodies.

Figure 4

Translation of a luciferase reporter gene plasmid is regulated by HELZ

(**A**) HeLa cells were transiently co-transfected with the indicated amounts of HELZ or a control expression vector together with pRL-SV40 renilla luciferase reporter vectors, and cultivated for 48 hours before relative luciferase activities were determined (upper panel). HELZ protein expression levels were controlled by immunoblotting (lower panel). (**B**) Renilla luciferase mRNA was measured by RT-qPCR from total RNA extracted in a duplicate experiment presented in (**A)**. Prior to RT, RNA was DNase-treated to digest possibly remaining plasmid DNA. Renilla mRNA levels were normalized to ribosomal protein L28 mRNA levels. (**C**) MCF-7 cells were transfected with the indicated siRNA oligonucleotides and 24 hours later with pRL-SV40, and cultivated for 48 hours before relative luciferase activities were determined (upper panel). HELZ knock-down efficiency was controlled by immunoblotting. Results are presented as mean values ± standard error of the mean of n = 3 (**A**) and n = 6 (**C**) independent experiments, respectively.

Figure 5

HELZ regulates global protein translation

(**A**) HeLa cells were transfected with 3 μg of the indicated constructs and 48 hours later pulse-labeled with [^{35}S]-methionine for 1 hour. Incorporated radioactivity was measured by scintillation counting following TCA precipitation and results were normalized to total protein levels (left panel). Protein overexpression was controlled by immunoblotting (right panel). (**B**) Schematic depiction of the different HELZ mutations (upper panel). HeLa cells were co-transfected with the indicated HELZ constructs together with pRL-SV40 and cultivated for 48 hours before luciferase activities were determined and normalized to total protein concentrations (lower left panel). Expression of V5-tagged constructs was controlled by immunoblotting (lower right panel). (**C**) HeLa cells were transfected with mock control, HELZ or HELZ in combination with PABP siRNA oligonucleotides and analyzed as described under (**B**). Expression of the transfected vectors and PABP knock-down efficiencies were

controlled by immunoblotting (right panel). Results are presented as mean values ± standard error of the mean of n = 3 independent experiments.

Figure 6

HELZ suppression limits cellular proliferation, ribosomal protein S6 phosphorylation and initiation of translation

(**A**) HeLa cells were transfected with the indicated siRNA oligonucleotides and cell numbers were assessed every 24 hours using an automated cell counter. (**B**) Immunoblot analysis of HeLa cells transfected with two different HELZ siRNA oligonucleotides and harvested at indicated time points after transfection (left panel). The experiment was performed three times independently and the pS6 band intensities were quantified 48 hours after transfection (right panel). (**C**) Protein levels of HELZ, p-S6 and total S6 in stable shRNA control (shctrl) or shRNA HELZ expressing cells were analyzed by immunoblotting. (**D**) Representative polysomal profile (n=3) of HeLa cells transfected with control or HELZ siRNA oligonucleotides.

Supporting Figure S1

Human hepatoma HuH7 cells were growth-arrested in G1 phase by incubation with 5 µg/ml aphidicolin for 36 hours and released from G1 phase by aphidicolin removal. At time points 0, 4, 8 or 12 hours later, cells were fixed with 4% (w/v) paraformaldehyde. Cell cycle progression verified by propidium iodide (PI) staining and FACS analysis (left panel). HELZ subcellular localization was visualized by indirect immunofluorescence and nuclei were stained with Dapi (right panel).

Supporting Figure S2

Crude HeLa cell lysates were incubated with recombinant GST, the indicated GST-HELZ fragments, GST-HIF-1$\alpha^{530-826}$ as well as GST-Paip2$^{106-127}$ and GST pull-down was conducted using glutathione-sepharose beads. Eluates were subjected to SDS-PAGE and immunoblotting.

Supporting Table 1

Overview of proteins with two conserved LxxLAP motifs

Using the PROSITE pattern search tool [53] the Swiss-Prot database was searched for proteins containing two LxxLAP motifs. Conservation analysis across 33 species was subsequently performed using the MULTIZ whole-genome multiple alignment algorithm [54] implemented in the UCSC Genome Browser [55].

Figures

Figure 1, Hasgall et al.

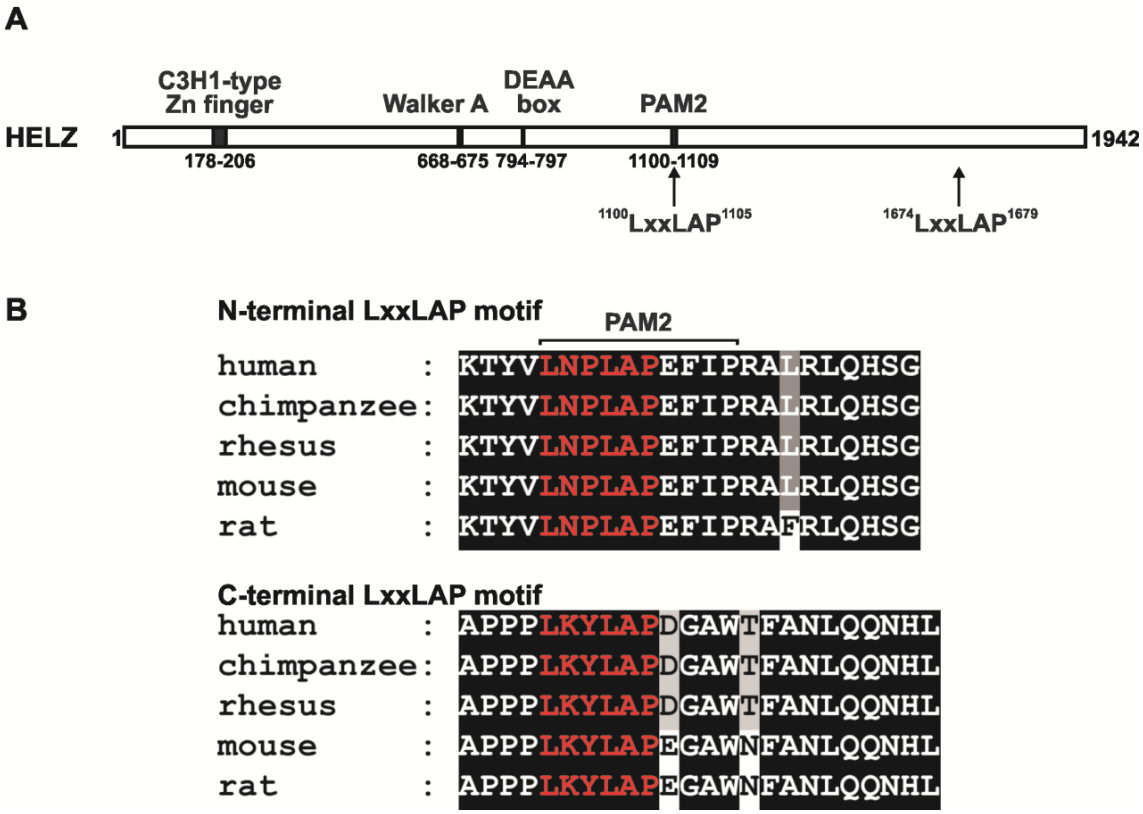


Figure 2, Hasgall et al.

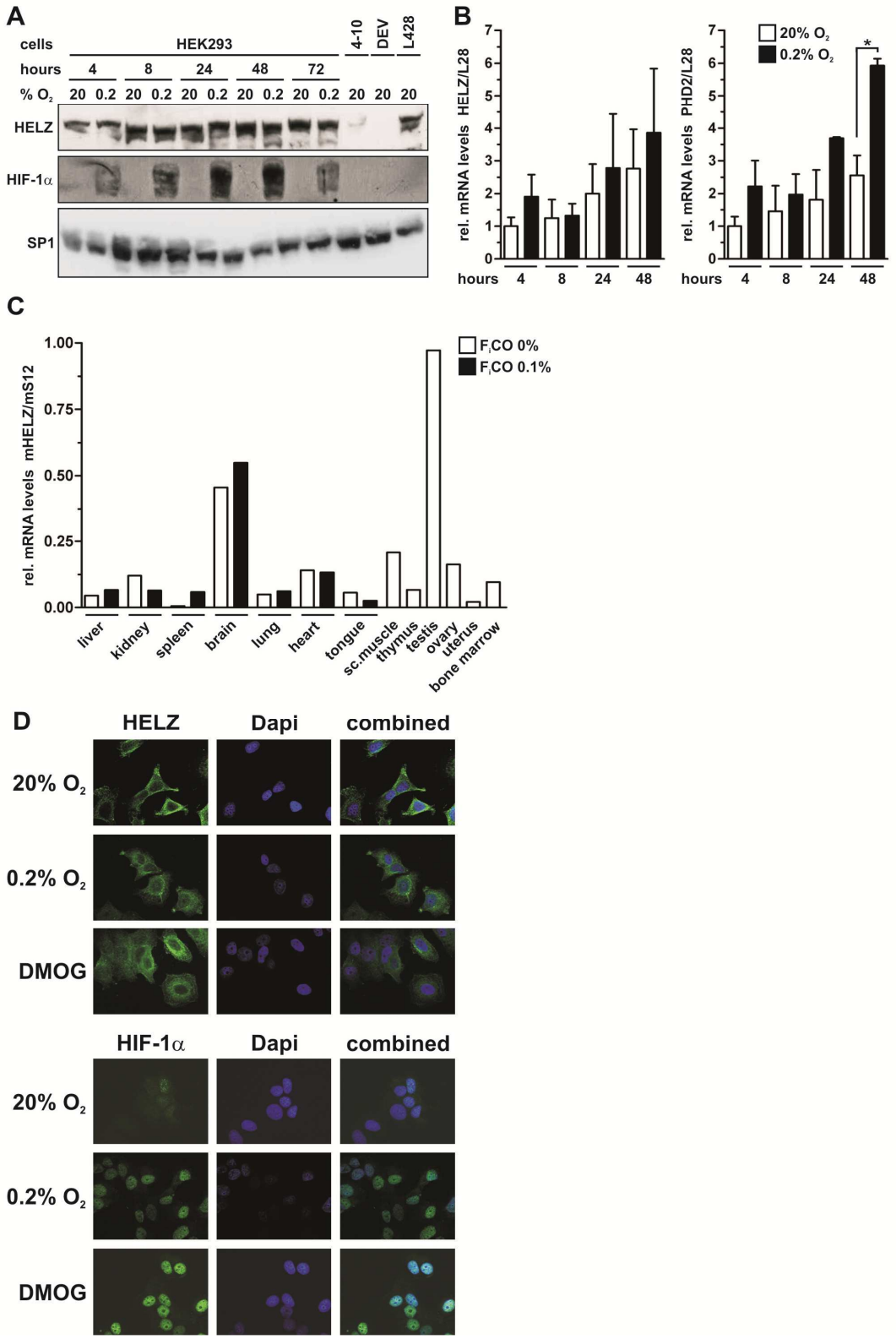


Figure 3, Hasgall et al.

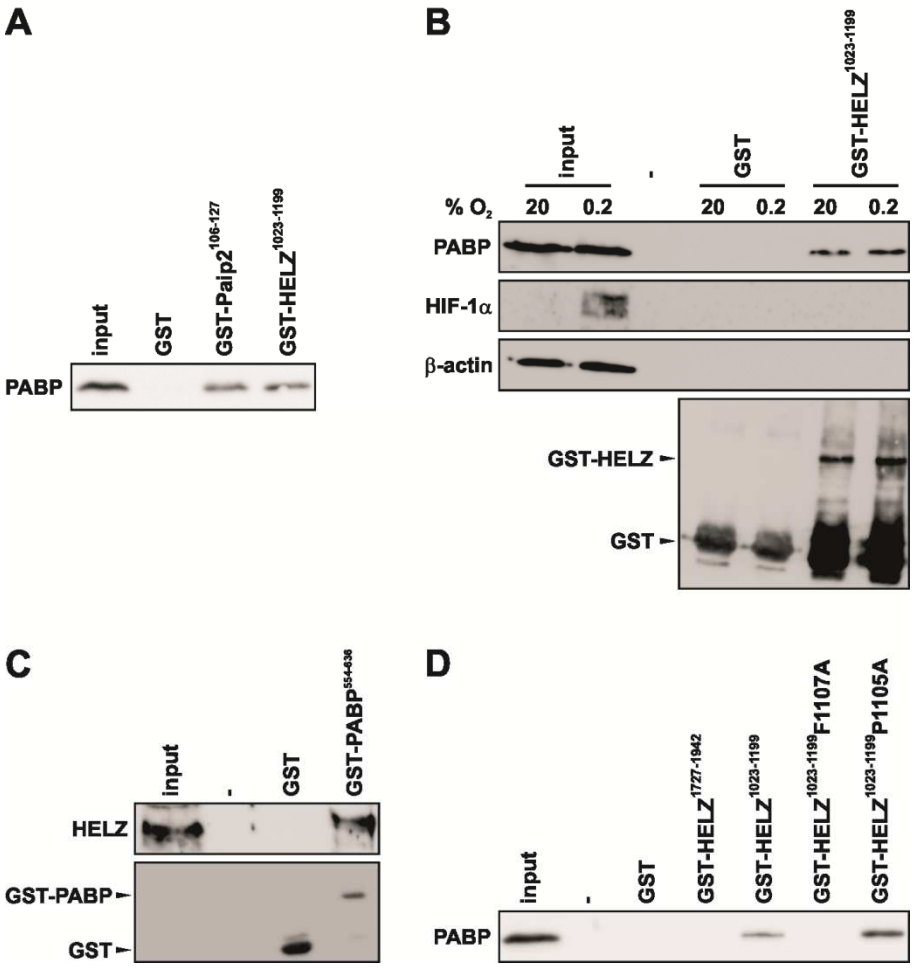


Figure 4, Hasgall et al.

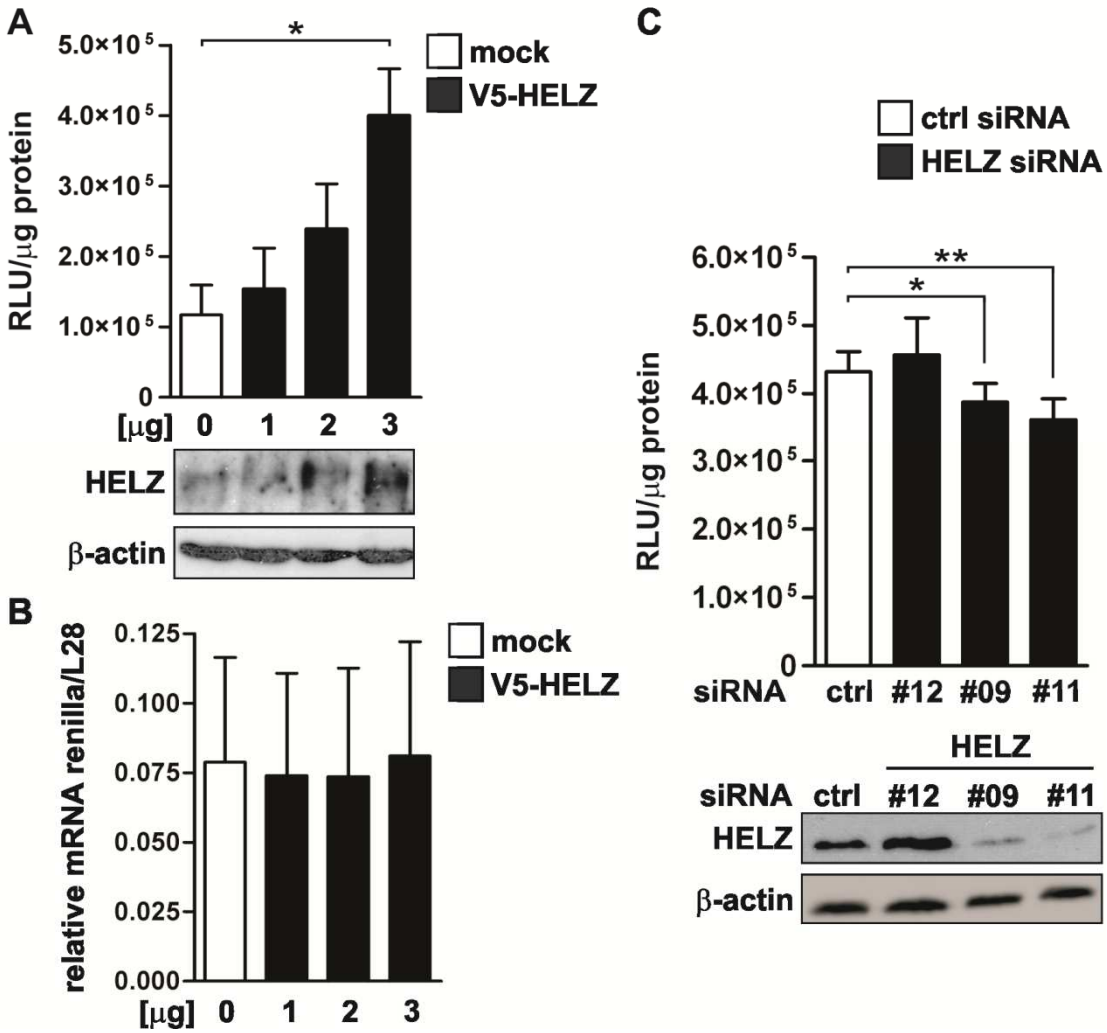


Figure 5, Hasgall et al.

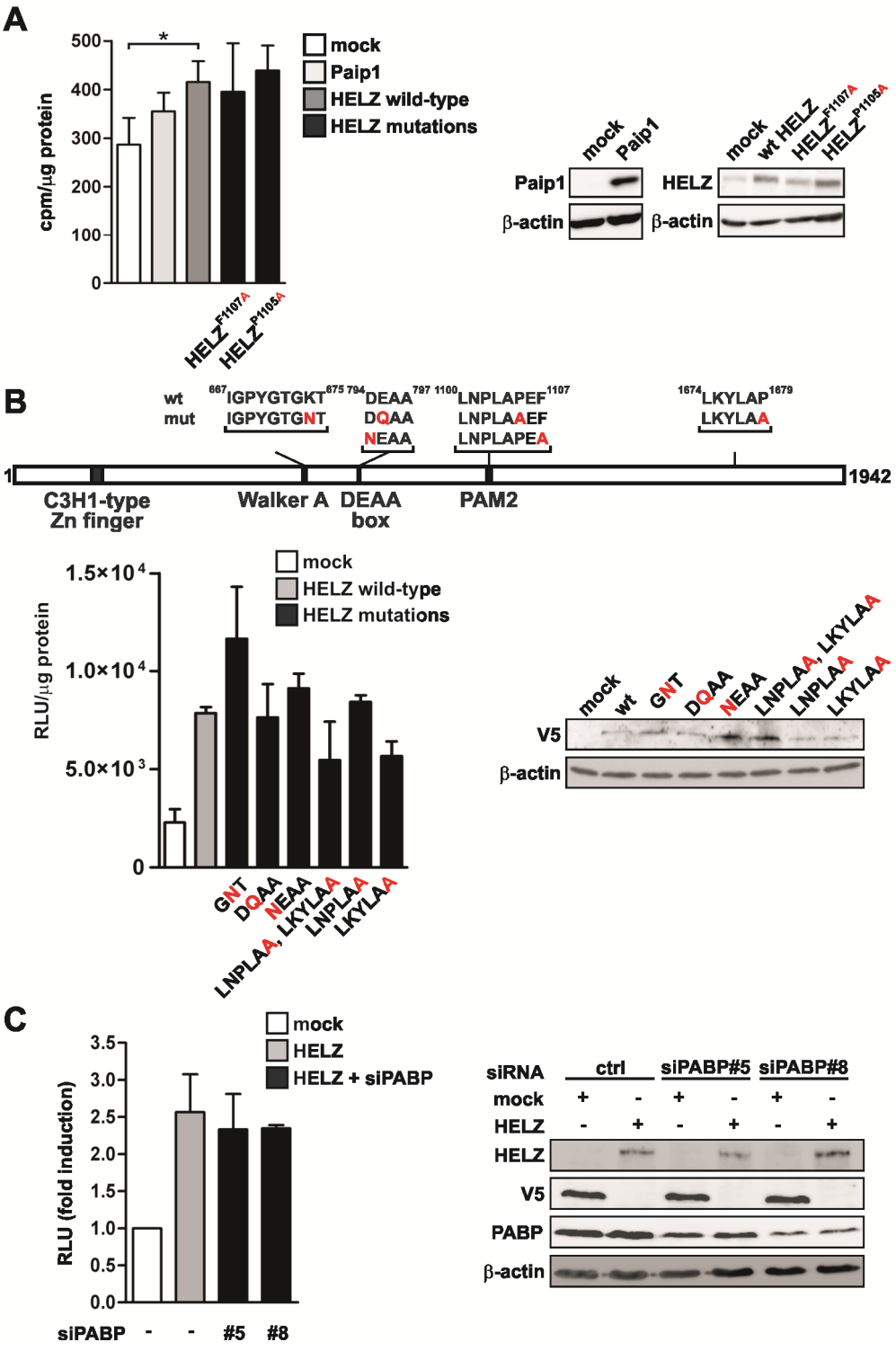
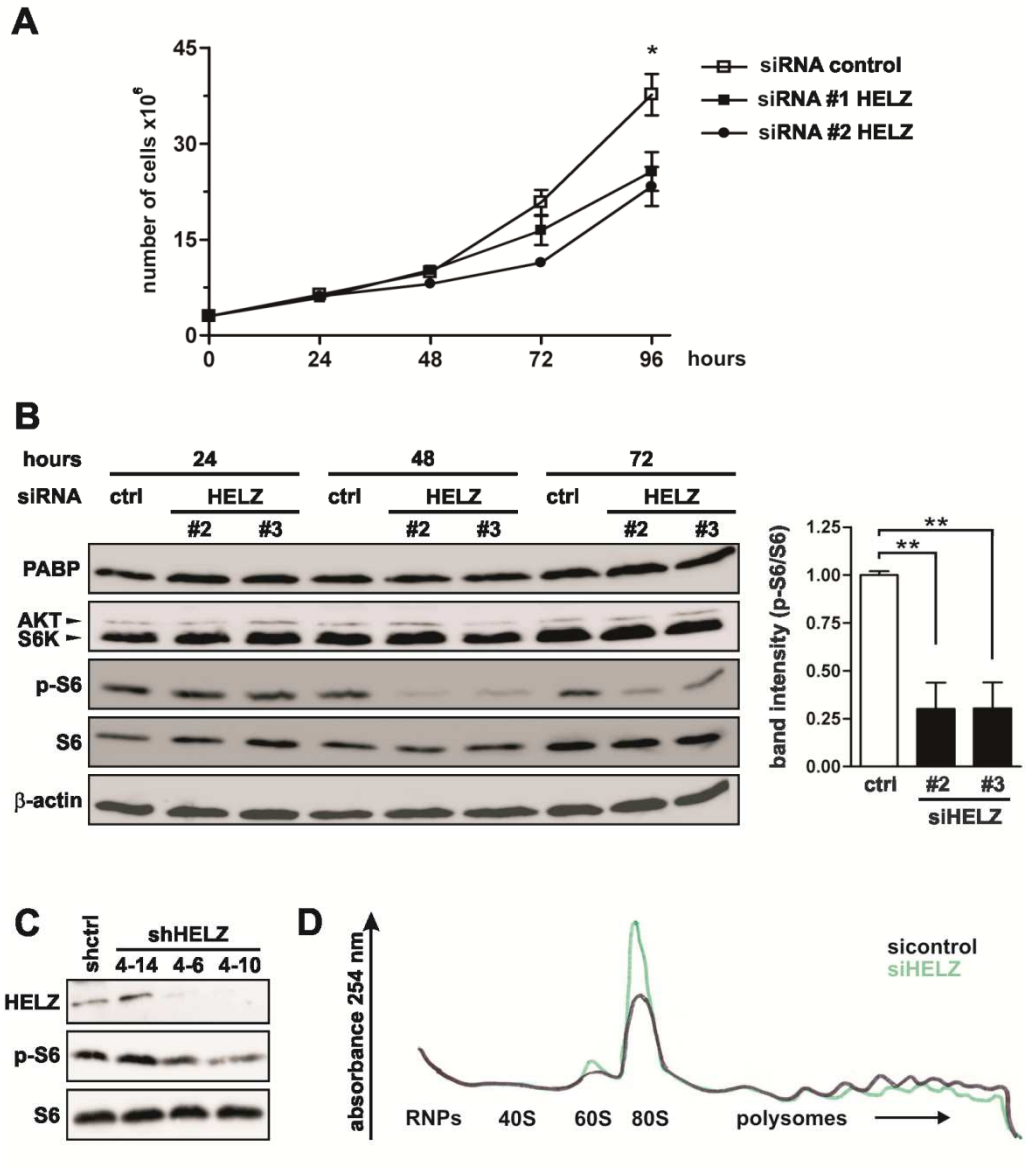
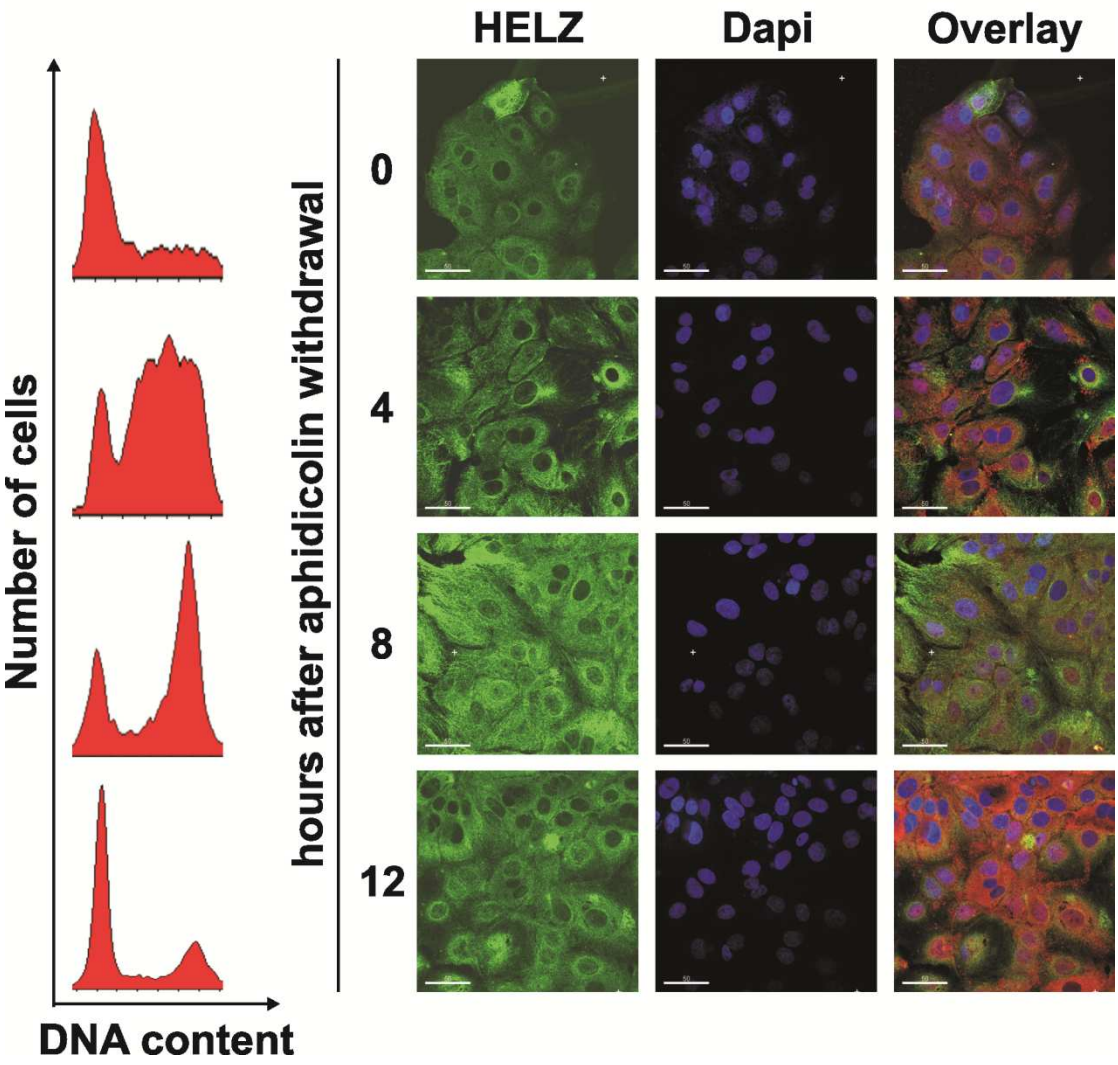


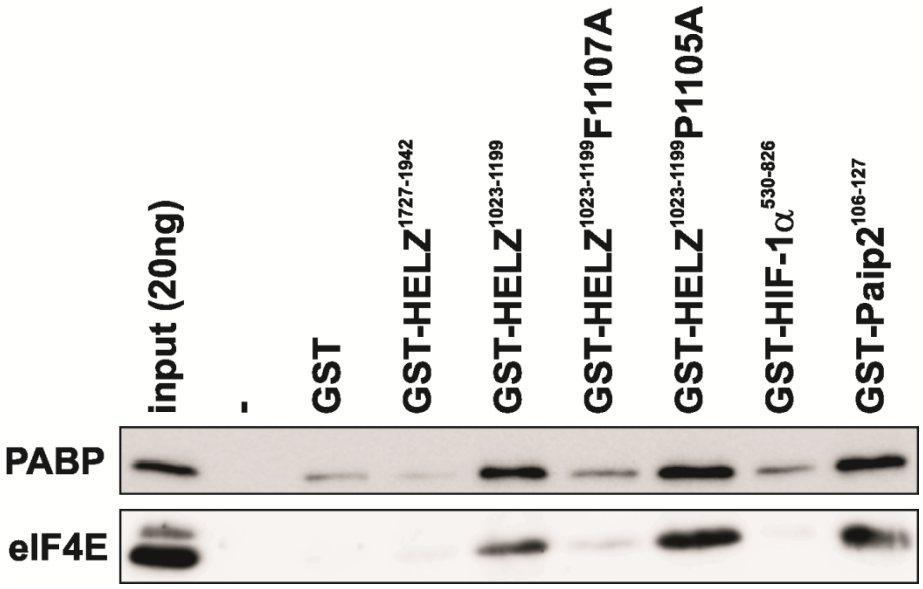
Figure 6, Hasgall et al.



Supporting Figure S1, Hasgall et al.



Supporting Figure S2, Hasgall et al.



Supporting Table 1, Hasgall et al.

		HELZ		HIF-1α		HIF-2α		CNOT1		FGD1	
LxxLAP:		LnpLAP	LkyLAP	LtlLAP	LemLAP	LaqLAP	LetLAP	LggLAP	LagLAP	LflLAP	LqkLAP
Position for human protein:		1100	1674	397	559	400	526	628	1367	265	457
Primates	*Human*										
	Chimp										
	Gorilla			-	-						-
	Orangutan		-								
	Rhesus										
	Baboon										
	marmoset										
	tarsier					-	-	-		-	
	mouse lemur		-				-				
	bushbaby					-		-		-	
Placental mammals	*tree shrew*										
	mouse					LaqQAT				LvrVAP	
	rat										
	kangaroo rat		-					-			
	guinea pig										
	squirrel							-	-		-
	rabbit								LagLSP		
	pika							-			
	alpaca					-				-	
	dolphin		LkcLAP					-			
	cow										
	horse										
	cat	-									
	dog										
	microbat		LryMEF						-		
	megabat	-									
	hedgehog		-				-		-		
	shrew		-			-	-				
	elephant										
	rock hyrax			-		-					-
	tenrec						-		LagPAP		
	armadillo	-									
	sloth							-			-

Part 5: Manuscript II

Characterization of novel HIF prolyl-4-hydroxylase 2 - interacting proteins

Philippe A. Hasgall, Roland H. Wenger, and Gieri Camenisch

Institute of Physiology and Zürich Center for Integrative Human Physiology ZIHP, University of Zürich UZH, CH-8057 Zürich, Switzerland

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; DMOG, dimethyloxalylglycine; GST, glutathione S-transferase; HELZ, helicase with zinc finger domain; HIF, hypoxia-inducible factor; ODD, oxygen-dependent degradation; PABP, poly(A)-binding protein; PHD, prolyl-4-hydroxylase domain enzyme; RT-qPCR, reverse transcription quantitative PCR; SG, stress granule; pVHL, von Hippel-Lindau tumor suppressor protein.

Abstract

Transcriptional activation of oxygen-dependent genes by heterodimeric hypoxia-inducible transcription factors (HIFs) is a crucial step in the adaptation of mammalian cells to low oxygen. HIF-α subunits are constantly expressed but the proteins rapidly degraded under normoxic conditions. A prerequisite for this oxygen-dependent degradation is the hydroxylation of specific prolyl residues by the prolyl-4-hydroxylase domain (PHD) enzymes. In hypoxia, prolyl hydroxylation reduction leads to stable HIF-α proteins and transcriptionally active HIF complexes. By applying yeast 2-hybrid screening to identify novel PHD2 interacting proteins we discovered three novel PHD2-interacting proteins: HELZ, ZBTB3 and KIAA0556. Direct interaction between these three proteins and PHD2 could not be confirmed in a GST pull-down assay, suggesting indirect interaction with PHD2 via a larger protein complex. Since HELZ is a RNA helicase we tested if HELZ affects the HIF transcriptional activity. HELZ did not affect HIF-dependent reporter gene regulation in the luciferase reporter cell line HRB5, suggesting that HELZ is not required for HIF-dependent transcriptional regulation. Even though HELZ interacts with the poly(A) binding protein it does not localize within stress granules after exposing cells to heat shock.

Introduction

Oxygen is a key substrate in cellular metabolism and insufficient oxygen availability can lead to cellular dysfunction or even cell death. To ensure adequate tissue oxygenation, aerobic organisms developed a variety of physiological responses to variation in oxygen availability. These involve the cardiovascular and respiratory system as well as cellular adaptation processes. Central to the cellular adaptation is the transcriptional activation of oxygen-regulated genes by the heterodimeric hypoxia-inducible transcription factor HIF that binds to a specific hypoxia-response element (HRE) on the DNA [109]. HIF-α and HIF-β proteins are continuously synthesized independent of oxygen tension, but the HIF-α protein undergoes oxygen-regulated degradation [3]. Under normoxic conditions two prolines within the ODD domain are hydroxylated by specific prolyl hydroxylases enabling the binding of the von Hippel-Lindau tumor suppressor protein (pVHL), a substrate recognition component of an E3 ubiquitin ligase complex, to the HIF-α subunit [3]. HIF-α gets ubiquitinated and subsequently targeted to the 26S proteasome for degradation [4, 5]. Under hypoxic conditions the prolyl hydroxylation reaction is inhibited, the interaction of pVHL with the HIF-α subunit abolished, resulting in HIF-α accumulation in the nucleus, dimerization with HIF-β, and induction of target gene expression [119, 125-127].

The proly-4-hydroxylase family consists of three members termed prolyl-4-hydroxylase domain (PHD) 1, PHD2 and PHD3 [6]. These three isoforms differ in their tissue expression and subcellular localization [149, 151]. It is postulated that PHD2 is the main regulator of HIF-α [7].

In the last few years a number of novel PHD interactors have been described. This includes novel PHD hydroxylation targets, but also proteins that regulate PHD function such as enzymatic activity or stability. For example, it has been shown by mass spectrometry analysis that the RING finger E3 ubiquitin ligase seven in absentia homolog 2 (SIAH2) interacts with the PHDs and that SIAH exhibits highest degree of binding with PHD3, but also associates strongly with PHD1 and to a much lesser extent with PHD2 [186]. In the same publication it has been shown that PHD3 stability is regulated by SIAH2 via targeting of PHD3 for proteasomal degradation [186]. Other proteins have been described to be novel PHD targets. For example,

siRNA-mediated *knock down* of PHD1 and PHD2 as well as treatment of cells with the PHD inhibitor DMOG resulted in NFκB induction [181]. Furthermore, overexpression of PHD1 decreased cytokine-stimulated NFκB reporter activity [181]. This PHD-dependent regulation of NFκB function might involve IKKβ since hypoxia increases its expression and activity. Interestingly, IKKβ contains an LxxLAP motif and site-directed mutagenesis of the proline residue resulted in loss of hypoxic inducibility, suggesting that IKKβ might be a novel PHD hydroxylation target. Another protein that has been shown to be hydroxylated in a PHD-dependent manner is Rpb1 [189]. Rpb1 can bind PHD1 and PHD2, and PHD1 was necessary for oxidative-stress-induced P1465 hydroxylation [178, 189]. Our laboratory recently discovered in a yeast 2-hybrid screen that FK506-binding protein (FKBP) 38, a peptidyl-prolyl cis/trans isomerase (PPIase), interacts specifically with PHD2 and functions as an adaptor protein mediating interaction of PHD2 with the proteasome leading to the degradation of PHD2 [187, 188].

Besides FKBP38, we identified helicase with zinc finger (HELZ), KIAA0556 and Zinc finger and BTB-domain containing (ZBTB) 3 as novel PHD2-interacting proteins. HELZ is putative RNA helicase and we previously reported that it interacts with the poly(A) binding protein (PABP) to promote protein translation leading to increased cell proliferation and ribosomal protein S6 phosphorylation [Hasgall et al., submitted for publication]. Furthermore, tagged-HELZ protein has been shown to co-immunoprecipitate with co-transfected histone methyltransferases Smyd2 and Smyd3 and a functional role as adaptor molecule to RNA polymerase II has been suggested [63, 64]. KIAA0556 and ZBTB3 have yet unknown functions.

In this report we analyze these interactions *in vitro* using biochemical as well as cellular interaction assays. We investigate the regulation of HELZ subcellular localization at different cell culture conditions and tested the involvement of HELZ in HIF transcriptional activity.

Results

Novel PHD2-interacting proteins

To identify novel PHD2-interacting proteins a yeast 2-hybrid screen of a human brain cDNA library using PHD2 as bait was performed. A total of $2x10^6$ individual transformants were screened and we found three proteins to interact with PHD2: HELZ, KIAA0556 and ZBTB3. In addition, the yeast 2-hybrid screens revealed that the interaction domain for HELZ is located between amino acid 1727 and 1942 and the interaction domain for KIAA0556 between amino acid 1098 and 1625. To confirm our findings in a yeast-independent interaction assay, we performed a GST pulldown assay. The novel PHD2-interacting proteins were recombinantly expressed as GST-fusion proteins (GST-HELZ$^{1727-1942}$, GST-KIAA05556$^{1098-1625}$ and GST-ZBTB3) in bacteria and incubated with IVTT-expressed ^{35}S-labeled PHD2. GST-HIF-1α$^{530-826}$ and GST-HIF-2α$^{404-569}$ served as positive controls and GST as negative control. Whereas the HIF-α fragments associated strongly with PHD2 the HELZ, KIAA0556 and ZBTB3 only showed a weak interaction signal suggesting that these proteins associated weakly or even indirectly via a protein complex with PHD2 (Figure 1A).

The most interesting of the three novel interactors was HELZ since it contains similar to HIF-α two conserved LxxLAP motifs [Hasgall et al., submitted for publication]. To confirm the interaction of HELZ with PHD2 and to test if HELZ also interacts with PHD1 and PHD3 in a mammalian system, we performed a mammalian 2-hybrid assay. p53, together with the polyomavirus coat protein (CP), served as a negative control and FKBP38, together with PHD2 served as positive control. Since the HIF-2α protein is only stable under hypoxic condition interaction with PHD2 can only be observed under hypoxic condition [187]. Since HELZ protein abundance may also be regulated hypoxically we tested the HELZ interactions with the PHDs under hypoxic and PHD-inhibiting conditions using the PHD-specific inhibitor DMOG. To our surprise no interaction could be observed between HELZ and PHD2 as well as with PHD1 and PHD3 (Figure 1C). Even though interaction of HIF-2αODD with PHD2 was greatly enhanced under hypoxic or PHD-inhibiting conditions, no interactions could be observed between HELZ and the three PHD isoforms. These findings suggest that HELZ does not interact with the PHDs in mammalian cells *in vivo*.

To test interaction of HELZ with PHD2 in a different mammalian system we performed a pull-down assay in HeLa cell lysates using recombinant GST-tagged HELZ fragments and anti-PHD2 antibodies allowing us to test interaction with the endogenous PHD2 protein. GST-HIF1$^{530-826}$ and GST alone served as positive and negative control, respectively. Furthermore, it has been shown that 20 residues long HIF-1α peptides suffice for both site-specific proline hydroxylation and subsequent binding to VHL [176]. In addition, the HELZ LxxLAP motifs do not reside within the C-terminal interaction domain. Hence, we tested if HELZ interacts with PHD2 through their highly conserved N-terminal LxxLAP motif. No interaction of HELZ with PHD2 was observed neither through the interaction site discovered in the yeast 2-hybrid screens nor through the LxxLAP motif (Figure 1C).

In summary, the interaction of HELZ with PHD2 observed in the Y2H was not confirmed in a mammalian system using overexpressed or recombinant protein. Furthermore, also the LxxLAP motif does not promote interaction with PHD2 suggesting that HELZ LxxLAP motifs may not be novel hydroxylation sites for PHD2.

HELZ involvement in HIF transcriptional activity

We previously demonstrated that HELZ protein levels are not hypoxically regulated [Hasgall et al, submitted for publication]. In a further attempt to demonstrate a role for HELZ in the context of hypoxia we performed a HIF-dependent reporter gene assay under hypoxic condition. For this purpose we performed a HELZ-specific transient *knock down* in HRB5 cells, a cell line derived from Hep3B that was stably transfected with a hypoxia responsive luciferase reporter construct [190]. *Knock down* of PHD2 served as positive control. Whereas siRNA mediated *knock down* of PHD2 induced HIF-dependent transcript even under normoxic condition HELZ *knock down* had no effect, neither under normoxic nor under hypoxic conditions (Figure 2A). *Knock down* efficiencies of HELZ and PHD2 were measured by RT-qPCR (Figure 2B). In summary, HELZ is not involved in hypoxia-induced, HIF-dependent gene transcription.

It has been shown previously in our group that stable *knock down* of PHD2 in MCF7 cells results in normoxic HIF-1α accumulation [191]. We were wondering if HELZ protein levels accumulate under the same conditions. For this purpose we measured

HELZ protein levels in PHD2-silenced MCF7 *knock-down* clones. HELZ protein did not accumulate in the clones suggesting that HELZ protein is not regulated by PHD2 similar to HIF-1α (Figure 2C).

HELZ subcellular localization

We recently demonstrated by immunofluorescence staining of MCF7 and Huh7 cells that HELZ is mainly expressed within the cytoplasm [Hasgall et al, submitted for publication]. To confirm this data with a different method, we performed biochemical fractionation of MCF7 and HEK293 cells into cytoplasmic and nuclear fractions and performed immunoblotting. Consistent with our immunofluorescence findings, HELZ protein was mainly expressed within the cytoplasm (Figure 3A). Immunostaining for HELZ in MCF7 and HEK293 cells also confirmed this data (Figure 3B).

Characterization of HELZ association with SMYD3

It has previously been shown that HELZ interacts with the methyl transferase SMYD3 [63]. In their study, HELZ was shown to be an adaptor protein bridging SMYD3 with the RNA polymerase II. However, no functional significance of this bridging function has yet been demonstrated. In an attempt to spread some light on the function of HELZ in the context of SMYD3 activity we tested the role of HELZ in the induction of the SMYD3 target genes. In a first step we overexpressed SMYD3 in HEK293 cells and measured the expression of the three SMYD3 target genes CCAAT/Enhancer Binding Protein (C/EBP) δ, Endothelin Converting Enzyme-Like (ECEL) 1 and Phosphoinositide-3-Kinase, catalytic, beta polypeptide (PIK3CB) by RT-qPCR. To our surprise none of the published SMYD3 target genes were significantly induced upon overexpression of SMYD3 (Figure 4A). Overexpression of SMYD3 was confirmed by immunoblotting (Figure 4A, bottom right panel).

In the same publication it was demonstrated by immunohistochemical staining of Huh7 cells with an anti-SMYD3 antibody that the subcellular localization of SMYD3 is altered by the density of the cell culture. In subconfluent cells, SMYD3 is expressed within the nucleus whereas in confluent cells within the cytoplasm. Since RNA helicases are known to shuttle between nucleus and cytoplasm [192-194] we were wondering if HELZ changes its subcellular localization similar to SMYD3. We

cultured Huh7 cells at different densities and measured HELZ localization by immunofluorescence using HELZ-specific antibodies. HELZ remained cytoplasmic independent of cell culture density (Figure 4B), suggesting that HELZ is not involved in the cell culture density-dependent alteration of SMYD3 subcellular localization.

HELZ and stress granules

In mammalian cells, different types of stress including ultraviolet (UV) irradiation, heat shock and oxidative stress inhibit translation of bulk mRNA, which aggregates in cytoplasmic structures known as stress granules (SGs) [195]. It has been shown that the poly(A) binding protein (PABP) resides within this stress granules during cellular stress such as heat shock [196]. To analyze whether HELZ might play a role in the context of stress granules we exposed HeLa cells to heat shock for two hours and detected HELZ subcellular localization by immunofluorescence. Whereas PABP was found within stress granules after heat stress HELZ was not (Figure 5). To our surprise, contrary to other tested cell lines, HELZ was localized in control cells within the nucleus as well as cytoplasm. Interestingly, after heat stress HELZ localized solely within the cytoplasm.

In summary, HELZ does not relocalize into stress granules during heat shock.

Discussion

A constant oxygen supply is crucial for the maintenance of an adequate energy metabolism since oxygen is the final electron acceptor in oxidative phosphorylation, the major process by which cells produces energy in the form of ATP. Central in the molecular adaptation to reduced oxygen supply is the hypoxia-inducible transcription factor HIF. HIF belongs to the basic helix-loop-helix/Per-ARNT-Sim (bHLH/PAS) family of transcription factors and is composed of a HIF-α and a HIF-β subunit. HIF-α and HIF-β proteins are continuously synthesized independently of oxygen, but the HIF-α proteins undergo oxygen-regulated degradation [3]. Central in this regulation is the function of a family of HIF proly-4-hydroxylases that use oxygen as substrate to hydroxylate two prolines (402 and 564) within the HIF-α protein, enabling pVHL binding resulting in HIF-α ubiquitination and proteasomal degradation [3-5]. Since the discovery of the PHDs, a lot of effort has been invested to find novel PHD-interacting proteins that might be novel hydroxylation targets or may act upstream of the PHDs. Rpb1, a RNA polymerase II subunit and IKKβ have been shown to be novel PHD hydroxylation targets [181, 189]. ATF 4 was found to interact with PHD3 and ATF4 protein stability were increased by hypoxia or PHD inhibition [185]. Upstream of PHD, the melanoma antigene gene protein (MAGE) 11 was found to inhibit PHD2 function without affecting PHD protein levels and our group found by using PHD2 as bait in a yeast 2-hybrid screen that FKBP38 interacts specifically with PHD2 and that it regulates PHD2 protein stability [187, 197]. In the same yeast 2-hybrid screen three other proteins were discovered to interact with PHD2: HELZ, KIAA0556 and ZBTB3. The interaction was confirmed by yeast retransformation. Interestingly, interaction could not be confirmed in a pull-down assay using recombinant proteins. This suggests that these novel PHD2-interacting proteins may not interact directly with PHD2, but are found in a protein complex together with PHD2. In this regard, it has been postulated that PHD2 is part of a large heteromeric complex [187]. Furthermore, PHD2 has been shown to coimmunoprecipitate with the RNA polymerase II subunit Rpb1, and an independent study showed that the same subunit interacts with exogenously-expressed HELZ protein [63, 189]. However, also using a mammalian 2-hybrid assay and pulldown assay in HeLa cell lysates did not confirm the interaction of HELZ with PHD2. Since the interaction observed in the

yeast 2-hybrid cannot be confirmed in a mammalian system the question arises if the observed yeast 2-hybrid interaction is a false positive or not. In our yeast 2-hybrid PHD2 was fused to a GAL4-DBD and HELZ to VP16-AD. It would be interesting to test if HELZ and PHD2 still interact if the tags would be exchanged fusing HELZ to the GAL4-DBD and PHD2 to the VP16-AD. Furthermore, yeast 2-hybrid interaction assay should be repeated in another yeast strain to exclude that the interaction of HELZ with PHD2 is strain specific. Besides our yeast 2-hybrid screen, HELZ has already been found in two different yeast 2-hybrid screens that were performed by two different groups independently [63, 198]. One of these groups actually found HELZ to be a false positive [198]. Furthermore, HELZ has been found to interact in mammalian cells with several proteins, namely SMYD3, SMYD2, RNA Polymerase II, and PABP and possibly with HYD since many PAM2-motif containing proteins interact with HYD [63, 64]. Proteins that have several interaction partners (e.g. heat shock proteins, ribosomal subunits) have specific structures that facilitate interaction with other proteins even when the interaction is not of functional significance. An exact determination of the three-dimensional structure of HELZ may reveal such structures. We show here that HELZ induces global translation. Expression of HELZ in yeast cells may therefore increase basal translation of the reporter genes used in our yeast 2-hybrid screen. Global protein translational rate should therefore be measured under the conditions used in our screen.

Adaptation to environmental changes is a great challenge for a cell since the changes are not always predictable. Cellular stress often results in translational arrest and the accumulation of untranslated mRNA in specific cytoplasmic foci termed stress granules (SGs) [199]. Only transcripts of dispensable mRNAs are stored within the granules, the translation of stress-induced transcripts such as for heat shock proteins is maintained or even enhanced [200]. Besides mRNA, these SGs usually contain members of the translation initiation machinery including the PABP [196]. We previously showed that HELZ interacts with PABP and that it is involved in the initiation of translation. Our data presented here indicate that HELZ does not accumulate within stress granules as shown by immunofluorescence. It would be interesting to test if HELZ plays a role in the translation of mRNAs that are involved in the adaptation to heat stress and are therefore not stored in stress granules. The change in subcellular localization during heat shock would suggest that HELZ plays an active role in the adaptation to heat stress.

In summary, in this work we show that the existence of even two highly conserved LxxLAP motifs cannot be taken as a prerequisite for the interaction with PHD2 and involvement in the HIF transcriptional activity. We show that HELZ, even though involved in translation initiation, does not translocate upon heat shock into stress granules suggesting that HELZ does not play a role in the surveillance of dispensable mRNAs during cellular stress.

Materials and methods

Unless otherwise indicated, cloning work was carried out using Gateway technology (Invitrogen, Basel, Switzerland) as described previously [187]. pDONR-HELZ$^{1727-1942}$, pDONR-KIAA0556$^{1098-1625}$ and pDONR-ZBTB3 originated from the yeast 2-hybrid screen as described previously [187]. The HELZ$^{1-1942}$ and SMYD3 cDNA was obtained from Origene (LabForce, Nunningen, Switzerland) and cloned into pENTR4 (Invitrogen). To generate fusion protein expression vectors, entry or donor vectors were recombined *in vitro* with destination vectors using LR Clonase recombination enzyme mix (Invitrogen). Glutathione S-transferase (GST) expression vector pDEST15 was used to generate GST-tagged fusion protein. The plasmids used to generate recombinant GST-HIF-1α$^{530-826}$ and recombinant GST-HIF-2α$^{404-569}$ were described previously [187, 201]. To generate expression plasmids for mammalian 2-hybrid analysis pM-DEST and pVP16-DEST were used [187].¨

Plasmids

Human MCF-7 breast carcinoma, human HeLa cervical carcinoma, human embryonic kidney HEK293 cells and HRB5 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Sigma, Buchs, Switzerland) as described previously [201], and human hepatoma HuH-7 cells were cultured in RPMI-1640 [202]. For hypoxic conditions, O_2 levels were decreased to 0.2% with N_2 in an oxygen-controlled incubator (Binder, Tuttlingen, Germany). Transfections were performed using polyethylenimine (Polysciences, Warrington, PA, USA) as described previously [162] or Lipofectamine2000 (Invitrogen). Cellular nuclear extracts and cytoplasmic fractions were prepared as previously described [203].

Yeast 2-hybrid

Yeast 2-hybrid screen was performed as described previously [187].

Reporter gene and mammalian 2-hybrid

The HIF-dependent firefly luciferase reporter cell line HRB5 was generated as described previously [190]. Cells were transfected with 100 nM of siRNA duplex

oligonucleotides using Lipofectamine 2000 (Invitrogen). 24 hours post-transfection cells were equally distributed and exposed to 20% or 0.2% oxygen for another 16 hours. Cells were washed twice in ice-cold PBS and luciferase activity was measured using the luciferase reporter assay system according to the manufacturer's instructions (Promega). Mammalian 2-hybrid analysis was performed as described previously [187].

Protein expression and purification

GST and GST fusion proteins were expressed in *Escherichia coli* BL21-AI by induction with 0.025% arabinose for 4 hours and affinity purified using glutathione-Sepharose beads (GSTrap FF; GE Healthcare, Dübendorf, Switzerland) according to the manufacturer's instructions.

GST pull-down

GST pull-down assay was performed as describe previously [187].

Immunoblotting

Immunoblot analyses were performed as described previously [204]. Antibodies used were mouse monoclonal antibody (mAb) anti-HELZ (Abnova, LucernaChem, Luzern, Switzerland), mAb anti-β-actin (Sigma), rabbit polyclonal antibody (pAb) anti-SP1 (Santa Cruz Biotechnology, LabForce, Nunningen, Switzerland), pAb anti-PHD2 (Novus Biologicals) and pAb anti-SMYD3 (Abcam).

mRNA quantification

Total cellular RNA was extracted as described previously [187]. First-strand cDNA synthesis was performed with 1 μg total RNA using reverse transcriptase (RT) and mRNA levels were measured by RT-qPCR using internal calibration standards and a SybrGreen qPCR reagent kit (Sigma) in combination with the MX3000P light cycler (Stratagene). To verify RNA integrity and equal input levels, ribosomal protein L28 mRNA was determined, and the data were expressed as ratios relative to L28 mRNA levels.

Immunofluorescence microscopy

Indirect immunofluorescence microscopy was performed as described previously [187].

Statistical analysis

Where not otherwise indicated, results are presented as mean values ± standard error of the mean of at least n = 3 independent experiments. All statistical tests were performed and graphed using GraphPad Prism v4.0 software (*, $P < 0.05$; **, $P < 0.01$).

References

1. Wenger, R. H., Stiehl, D. P. and Camenisch, G. (2005) *Integration of oxygen signaling at the consensus HRE.* Sci STKE, **2005**(306): p. re12.
2. Maxwell, P. H., *et al.* (1999) *The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis.* Nature, **399**(6733): p. 271-5.
3. Kallio, P. J., Wilson, W. J., O'Brien, S., Makino, Y. and Poellinger, L. (1999) *Regulation of the hypoxia-inducible transcription factor 1alpha by the ubiquitin-proteasome pathway.* J Biol Chem, **274**(10): p. 6519-25.
4. Salceda, S. and Caro, J. (1997) *Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes.* J Biol Chem, **272**(36): p. 22642-7.
5. Jewell, U. R., Kvietikova, I., Scheid, A., Bauer, C., Wenger, R. H. and Gassmann, M. (2001) *Induction of HIF-1alpha in response to hypoxia is instantaneous.* Faseb J, **15**(7): p. 1312-4.
6. Kallio, P. J., Okamoto, K., O'Brien, S., Carrero, P., Makino, Y., Tanaka, H. and Poellinger, L. (1998) *Signal transduction in hypoxic cells: inducible nuclear translocation and recruitment of the CBP/p300 coactivator by the hypoxia-inducible factor-1alpha.* Embo J, **17**(22): p. 6573-86.
7. Kallio, P. J., Pongratz, I., Gradin, K., McGuire, J. and Poellinger, L. (1997) *Activation of hypoxia-inducible factor 1alpha: posttranscriptional regulation and conformational change by recruitment of the Arnt transcription factor.* Proc Natl Acad Sci U S A, **94**(11): p. 5667-72.
8. Chilov, D., Camenisch, G., Kvietikova, I., Ziegler, U., Gassmann, M. and Wenger, R. H. (1999) *Induction and nuclear translocation of hypoxia-inducible factor-1 (HIF-1): heterodimerization with ARNT is not necessary for nuclear accumulation of HIF-1alpha.* J Cell Sci, **112 (Pt 8)**: p. 1203-12.
9. Epstein, A. C., *et al.* (2001) *C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation.* Cell, **107**(1): p. 43-54.
10. Lieb, M. E., Menzies, K., Moschella, M. C., Ni, R. and Taubman, M. B. (2002) *Mammalian EGLN genes have distinct patterns of mRNA expression and regulation.* Biochem Cell Biol, **80**(4): p. 421-6.
11. Soilleux, E. J., Turley, H., Tian, Y. M., Pugh, C. W., Gatter, K. C. and Harris, A. L. (2005) *Use of novel monoclonal antibodies to determine the expression and distribution of the hypoxia regulatory factors PHD-1, PHD-2, PHD-3 and FIH in normal and neoplastic human tissues.* Histopathology, **47**(6): p. 602-10.
12. Berra, E., Benizri, E., Ginouves, A., Volmat, V., Roux, D. and Pouyssegur, J. (2003) *HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1alpha in normoxia.* Embo J, **22**(16): p. 4082-90.
13. Nakayama, K., *et al.* (2004) *Siah2 regulates stability of prolyl-hydroxylases, controls HIF1alpha abundance, and modulates physiological responses to hypoxia.* Cell, **117**(7): p. 941-52.

14. Cummins, E. P., *et al.* (2006) *Prolyl hydroxylase-1 negatively regulates IkappaB kinase-beta, giving insight into hypoxia-induced NFkappaB activity.* Proc Natl Acad Sci U S A, **103**(48): p. 18154-9.
15. Mikhaylova, O., *et al.* (2008) *The von Hippel-Lindau tumor suppressor protein and Egl-9-Type proline hydroxylases regulate the large subunit of RNA polymerase II in response to oxidative stress.* Mol Cell Biol, **28**(8): p. 2701-17.
16. Kuznetsova, A. V., Meller, J., Schnell, P. O., Nash, J. A., Ignacak, M. L., Sanchez, Y., Conaway, J. W., Conaway, R. C. and Czyzyk-Krzeska, M. F. (2003) *von Hippel-Lindau protein binds hyperphosphorylated large subunit of RNA polymerase II through a proline hydroxylation motif and targets it for ubiquitination.* Proc Natl Acad Sci U S A, **100**(5): p. 2706-11.
17. Barth, S., *et al.* (2007) *The peptidyl prolyl cis/trans isomerase FKBP38 determines hypoxia-inducible transcription factor prolyl-4-hydroxylase PHD2 protein stability.* Mol Cell Biol, **27**(10): p. 3758-68.
18. Barth, S., Edlich, F., Berchner-Pfannschmidt, U., Gneuss, S., Jahreis, G., Hasgall, P. A., Fandrey, J., Wenger, R. H. and Camenisch, G. (2009) *Hypoxia-inducible factor prolyl-4-hydroxylase PHD2 protein abundance depends on integral membrane anchoring of FKBP38.* J Biol Chem, **284**(34): p. 23046-58.
19. Hamamoto, R., Furukawa, Y., Morita, M., Iimura, Y., Silva, F. P., Li, M., Yagyu, R. and Nakamura, Y. (2004) *SMYD3 encodes a histone methyltransferase involved in the proliferation of cancer cells.* Nat Cell Biol, **6**(8): p. 731-40.
20. Diehl, F., *et al.* (2010) *Cardiac deletion of Smyd2 is dispensable for mouse heart development.* PLoS One, **5**(3): p. e9748.
21. Huang, J., Zhao, Q., Mooney, S. M. and Lee, F. S. (2002) *Sequence determinants in hypoxia-inducible factor-1alpha for hydroxylation by the prolyl hydroxylases PHD1, PHD2, and PHD3.* J Biol Chem, **277**(42): p. 39792-800.
22. Martin, F., *et al.* (2005) *Copper-dependent activation of hypoxia-inducible factor (HIF)-1: implications for ceruloplasmin regulation.* Blood, **105**(12): p. 4613-9.
23. Bordoli, M. R., Stiehl, D. P., Borsig, L., Kristiansen, G., Hausladen, S., Schraml, P., Wenger, R. H. and Camenisch, G. (2010) *Prolyl-4-hydroxylase PHD2- and hypoxia-inducible factor 2-dependent regulation of amphiregulin contributes to breast tumorigenesis.* Oncogene.
24. Reed, R. and Hurt, E. (2002) *A conserved mRNA export machinery coupled to pre-mRNA splicing.* Cell, **108**(4): p. 523-31.
25. Nakielny, S. and Dreyfuss, G. (1999) *Transport of proteins and RNAs in and out of the nucleus.* Cell, **99**(7): p. 677-90.
26. Kohler, A. and Hurt, E. (2007) *Exporting RNA from the nucleus to the cytoplasm.* Nat Rev Mol Cell Biol, **8**(10): p. 761-73.
27. Eulalio, A., Behm-Ansmant, I. and Izaurralde, E. (2007) *P bodies: at the crossroads of post-transcriptional pathways.* Nat Rev Mol Cell Biol, **8**(1): p. 9-22.
28. Kedersha, N. L., Gupta, M., Li, W., Miller, I. and Anderson, P. (1999) *RNA-binding proteins TIA-1 and TIAR link the phosphorylation of eIF-2 alpha to the assembly of mammalian stress granules.* J Cell Biol, **147**(7): p. 1431-42.
29. Aprelikova, O., Pandolfi, S., Tackett, S., Ferreira, M., Salnikow, K., Ward, Y., Risinger, J. I., Barrett, J. C. and Niederhuber, J. (2009) *Melanoma antigen-11*

inhibits the hypoxia-inducible factor prolyl hydroxylase 2 and activates hypoxic response. Cancer Res, **69**(2): p. 616-24.

30. Shimomura, Y., Aoki, N., Ito, K. and Ito, M. (2003) *Gene expression of Sh3d19, a novel adaptor protein with five Src homology 3 domains, in anagen mouse hair follicles.* J Dermatol Sci, **31**(1): p. 43-51.
31. Buchan, J. R. and Parker, R. (2009) *Eukaryotic stress granules: the ins and outs of translation.* Mol Cell, **36**(6): p. 932-41.
32. Fonager, J., Beedholm, R., Clark, B. F. and Rattan, S. I. (2002) *Mild stress-induced stimulation of heat-shock protein synthesis and improved functional ability of human fibroblasts undergoing aging in vitro.* Exp Gerontol, **37**(10-11): p. 1223-8.
33. Kimball, S. R., Horetsky, R. L., Ron, D., Jefferson, L. S. and Harding, H. P. (2003) *Mammalian stress granules represent sites of accumulation of stalled translation initiation complexes.* Am J Physiol Cell Physiol, **284**(2): p. C273-84.
34. Camenisch, G., Tini, M., Chilov, D., Kvietikova, I., Srinivas, V., Caro, J., Spielmann, P., Wenger, R. H. and Gassmann, M. (1999) *General applicability of chicken egg yolk antibodies: the performance of IgY immunoglobulins raised against the hypoxia-inducible factor 1alpha.* Faseb J, **13**(1): p. 81-8.
35. Popowski, K., Eloranta, J. J., Saborowski, M., Fried, M., Meier, P. J. and Kullak-Ublick, G. A. (2005) *The human organic anion transporter 2 gene is transactivated by hepatocyte nuclear factor-4 alpha and suppressed by bile acids.* Mol Pharmacol, **67**(5): p. 1629-38.
36. Stiehl, D. P., Wirthner, R., Koditz, J., Spielmann, P., Camenisch, G. and Wenger, R. H. (2006) *Increased prolyl 4-hydroxylase domain proteins compensate for decreased oxygen levels. Evidence for an autoregulatory oxygen-sensing system.* J Biol Chem, **281**(33): p. 23482-91.
37. Chilov, D., Hofer, T., Bauer, C., Wenger, R. H. and Gassmann, M. (2001) *Hypoxia affects expression of circadian genes PER1 and CLOCK in mouse brain.* Faseb J, **15**(14): p. 2613-22.
38. Balamurugan, K., *et al.* (2009) *Onconeuronal cerebellar degeneration-related antigen, Cdr2, is strongly expressed in papillary renal cell carcinoma and leads to attenuated hypoxic response.* Oncogene, **28**(37): p. 3274-85.

Figure legends

Figure 1: HELZ, KIAA0556 and ZBTB3 interaction with PHD2

(A) IVTT ^{35}S-labeled PHD2 was incubated with GST alone, GST-HIF-1α$^{530-826}$, GST-HIF-2α$^{404-569}$, GST-KIAA0556$^{1098-1625}$, GST-ZBTB3 or GST-HELZ$^{1727-1942}$. Bound proteins were pulled down and visualized by phosphorimaging.

(B) HeLa cells were transiently transfected with Gal4-DBD and VP16AD fusion protein vectors and a Gal4 response element-driven firefly luciferase reporter, as well as a renilla luciferase control vector. 36 hours post transfection cells were exposed to 20% O_2, 0.2% O_2 or 2 μM DMOG and luciferase reporter gene activities were determined 18 hours later. Mean values plus standard errors of the mean are shown for three independent experiments performed in triplicate.

(C) GST pull-down using glutathione-sepharose beads was conducted by incubating crude HeLa cell lysates with recombinant GST, GST-HELZ$^{1727-1942}$, GST-HELZ$^{1023-1199}$, GST-HELZ$^{1023-1199}$F1107A, GST-HELZ^{10231199}P1105A and GST-HIF-1α.

Figure 2: HELZ involvement in HIF-transcriptional activity

(A) HRB5 cells were transiently transfected with the indicated siRNA oligonucleotides. HRE-reporter activity was determined by measuring luciferase activity and normalization to protein concentrations.

(B) *Knock-down* efficiency was tested by measuring HELZ and PHD2 mRNA levels in the HELZ or PHD2-silenced HRB5 cells using RT-qPCR.

(C) HELZ protein levels were measured in MCF7 wild-type cells (wt) and stable PHD2-silenced MCF7 clones by immunoblotting using anti-HELZ antibodies. *Knock-down* efficiently was tested with anti-PHD2 antibodies and β-actin was used as loading control. Two independent experiments are shown.

Figure 3: HELZ subcellular localization

(A) Cytoplasmic fractions (C), nuclear extracts (N) and whole cell extracts (WCE) were separated by SDS PAGE followed by immunoblotting and detection with anti-HELZ antibodies. The stable HELZ shRNA clone 4-10 and the Hodgkin's lymphoma-derived cell line DEV containing a homozygous deletion on chromosome 17 served as

controls for reduced and absent HELZ protein levels, respectively. Following incubation with the anti-HELZ antibody the membrane was incubated with anti-Sp1 and anti-β-actin control antibodies.

(B) Human hepatoma Huh7 cells were culture at the indicated concentration and fixed with 4% of paraformaldehyde. HELZ subcellular localization was visualized by indirect immunofluorescence and nuclei were stained with DAPI.

Figure 4: SMYD3 target gene expression

(A) HEK293 cells were transiently co-transfected with the indicated amounts of SMYD3 or a control expression vector and mRNA levels of the indicated SMYD3 target genes was measured by RT-qPCR. SMYD3 overexpression efficiency was measured by immunoblotting.

(B) HEK293 cells were culture at the indicated cell culture densities and fixed with 4% paraformaldehyde. HELZ subcellular localization was visualized by indirect immunofluorescence and nuclei were stained with DAPI.

Figure 5: Effect of heat shock on HELZ subcellular localization

HeLa cells were heat shocked (46°C) for 2 hours and subsequently fixed in 4% paraformaldehyde. HELZ and PABP subcellular localization was visualized by indirect immunofluorescence and nuclei were stained with DAPI.

Figures

Figure 1, Hasgall et al.

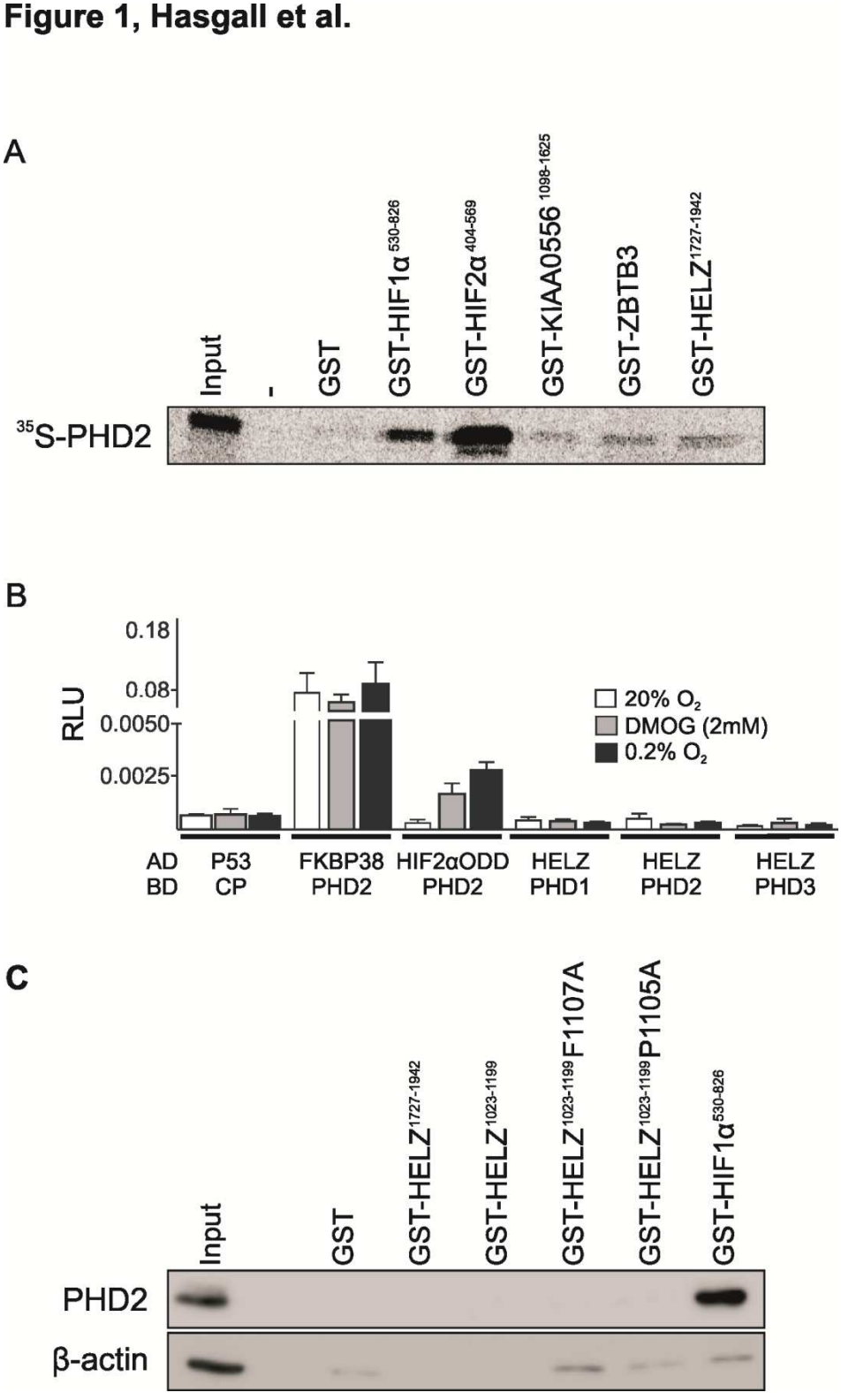


Figure 2, Hasgall et al.

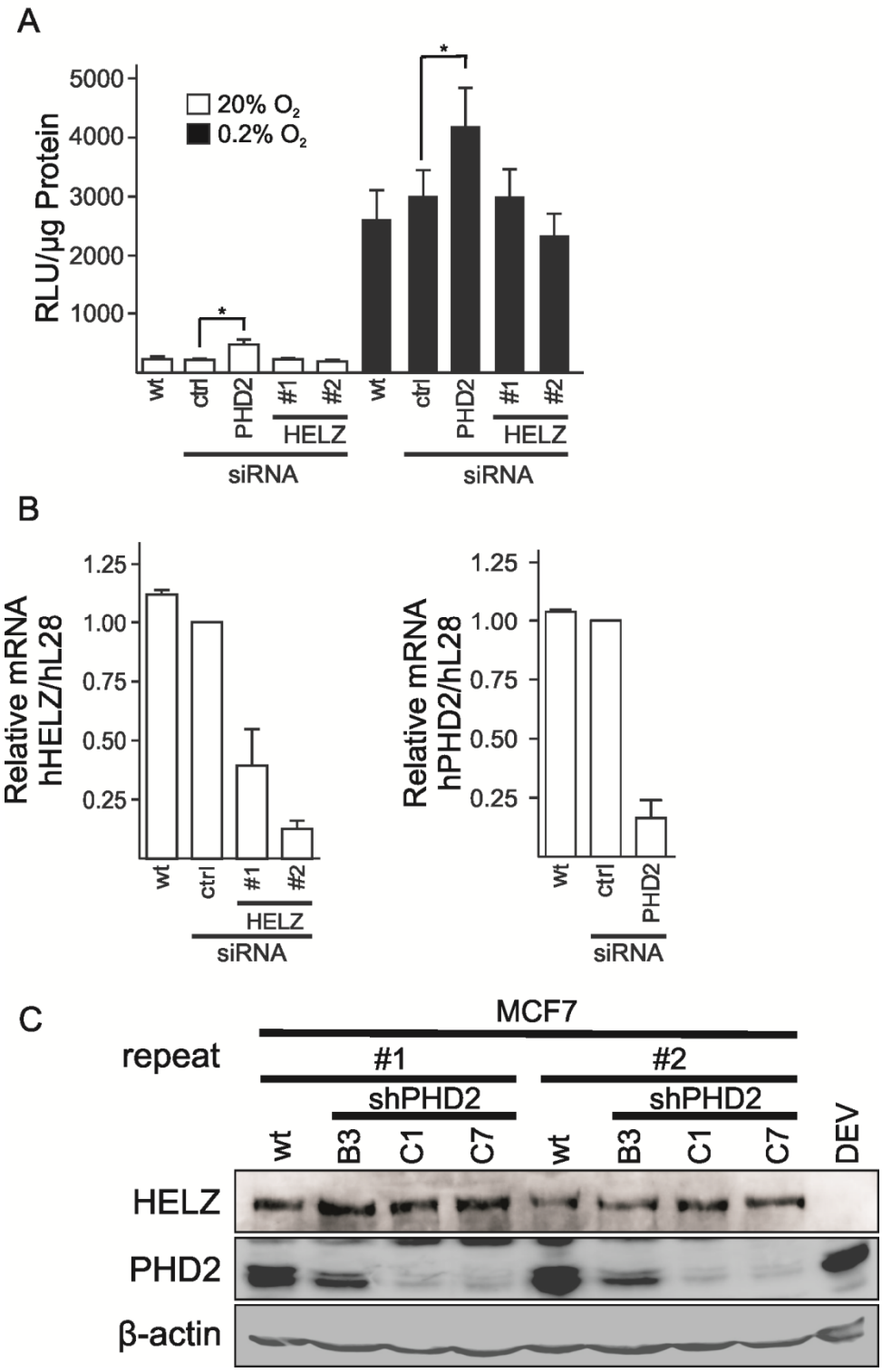


Figure 3, Hasgall et al.

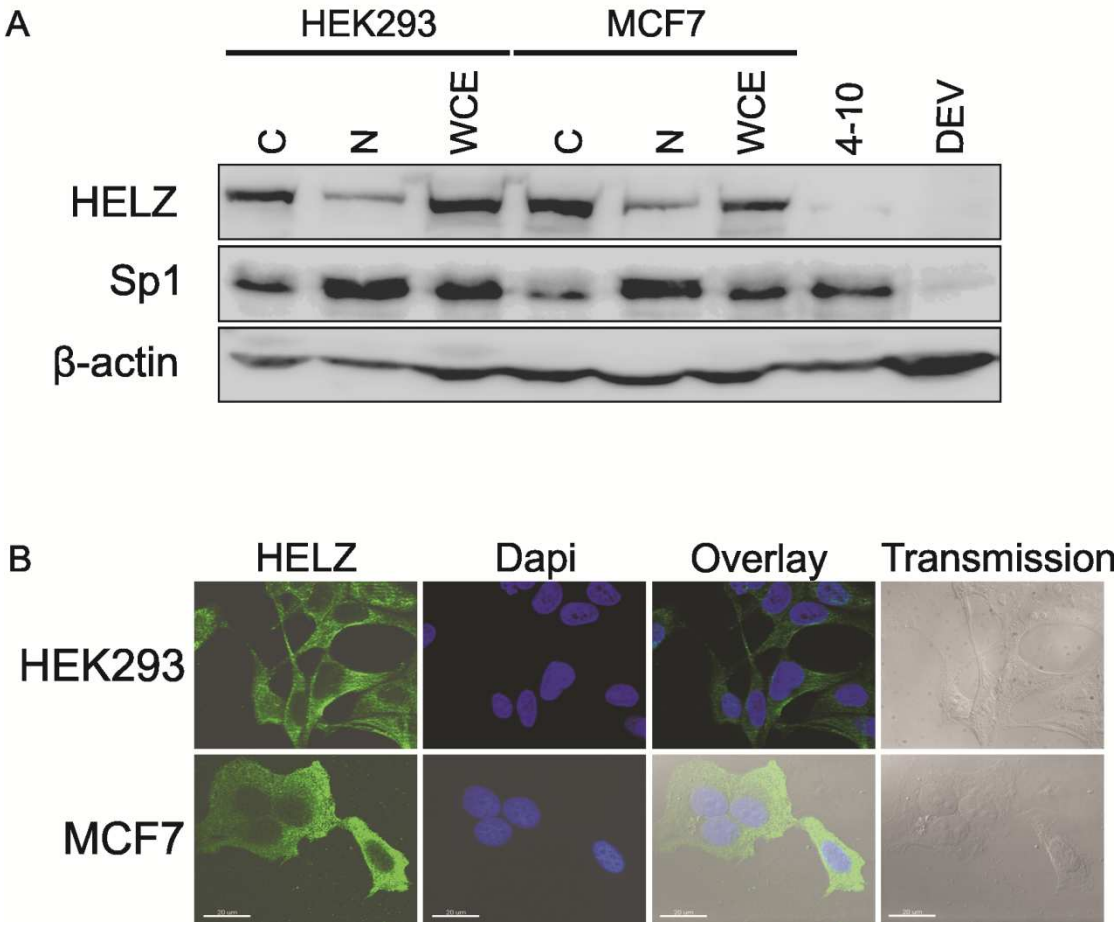


Figure 4, Hasgall et al.

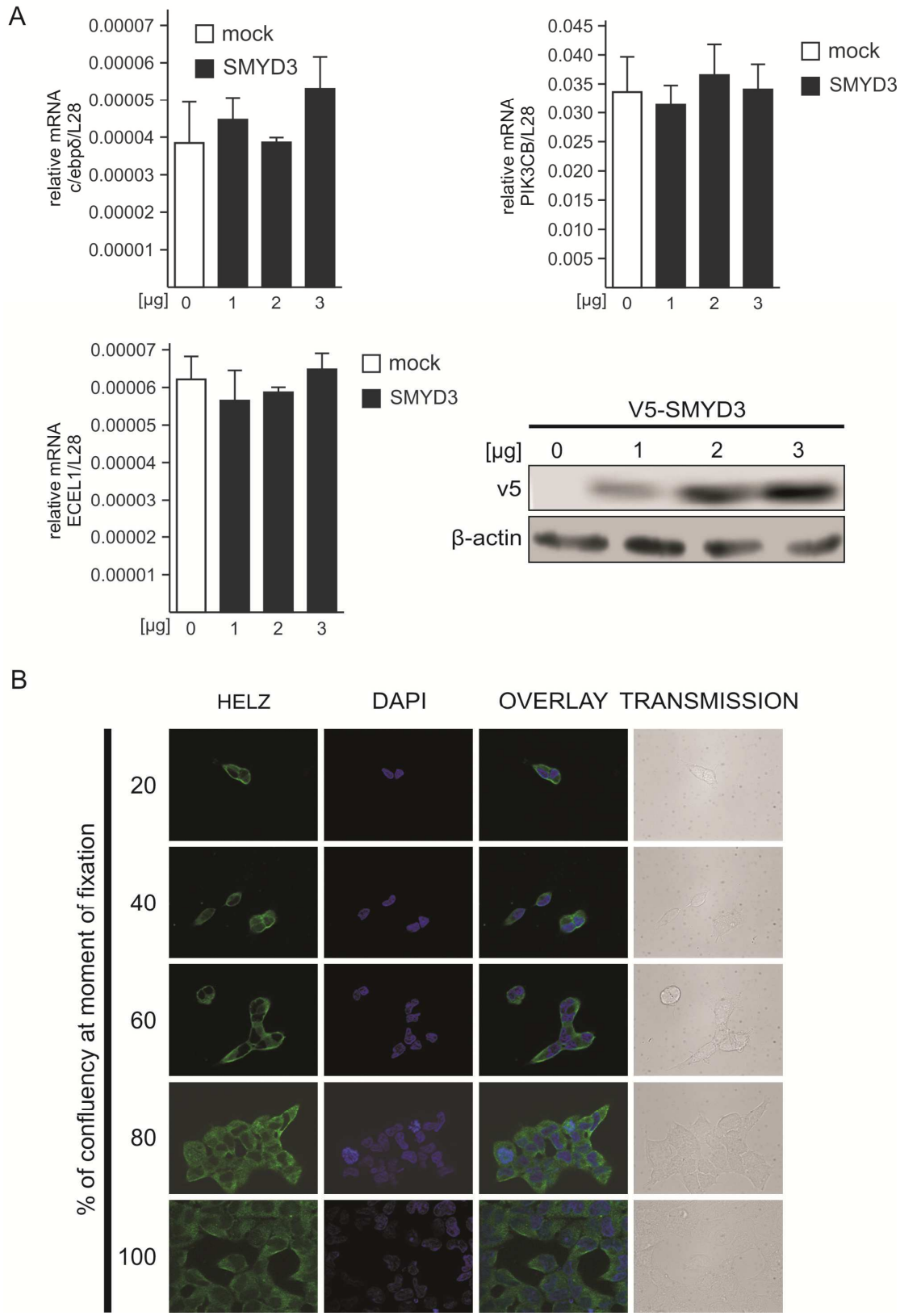
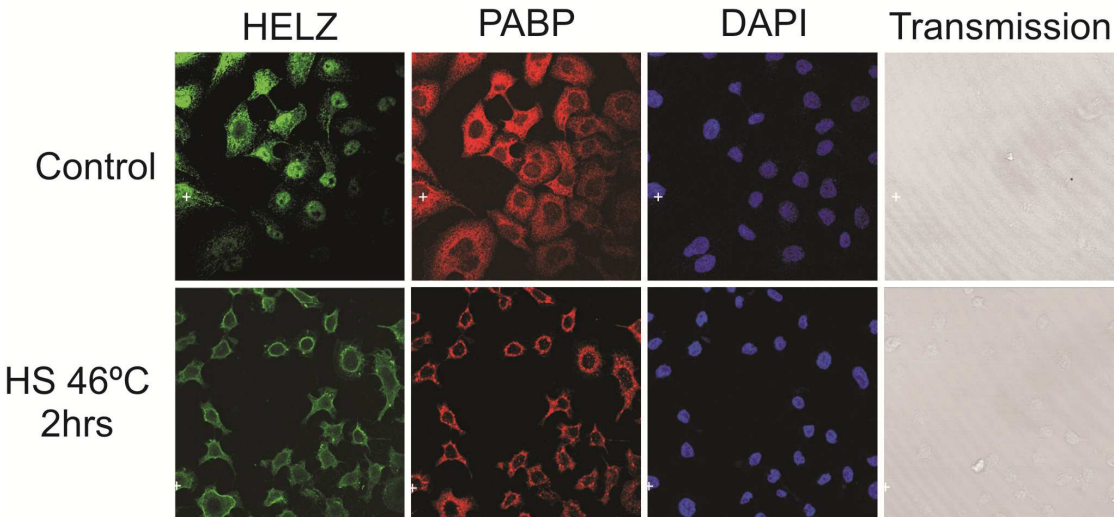


Figure 5, Hasgall et al.



Part 6: Discussion

Protein expression is one way how cells regulate protein activity. Protein expression is tightly regulated on several levels including transcription, translation and also post-translational modifications. The hypoxia-inducible factor HIF is strongly regulated on its post-translational level through a family of prolyl-4-hydroxylases (PHDs) that hydroxlyate the HIF-α subunit at specific prolines resulting in the destruction of the HIF-α protein in the proteasome. Under low oxygen conditions, PHD function is impaired leading to a stable HIF-α subunit resulting in the formation of a transcriptionally active HIF protein. Since the discovery of the PHDs the question arose if the PHDs have hydroxylation targets other than HIF-α or if PHD interaction partners exist that regulate the PHD catalytic activity or abundance. To find such novel PHD interactors, we performed a yeast 2-hybrid screen using PHD2 as bait. There are three PHD isoforms, PHD1, PHD2 and PHD3 and we decided to center our focus on PHD2, since it has been shown that it is the major regulator of the HIF-α subunit [6, 7]. We discovered three novel PHD2-interacting proteins: helicase with zinc finger domain (HELZ), zinc finger and BTB domain containing protein (ZBTB) 3 and KIAA0556.

In the HIF-α protein both hydroxyl prolines are located within a LxxLAP motif [175]. Besides HIF-α, two other proteins have been described to be hydroxylated in the context of a LxxLAP motif namely Rpb1 and IκB kinase-β [181, 189]. We used bioinformatic research tools to analyse the novel interactors for LxxLAP motifs and found that HELZ contains two evolutionary conserved LxxLAP motifs. ZBTB3 and KIAA0556 do not contain any LxxLAP motifs. Based on this observation we decided to keep our main focus on HELZ. HIF-α interacts with all three PHDs and we also studied the interaction of HELZ with PHD1, PHD2 and PHD3. However, none of the interaction assays used in this study could clearly demonstrate the association of HELZ with any of the PHDs in mammalian cell systems. This is surprising, but could be explained by the fact that we performed most of our assays under normoxic conditions and it is possible that under these conditions the HELZ protein is not stable and interaction can therefore not be detected. The existence of the two LxxLAP motifs would support such an idea. To investigate this option, we tested HELZ protein expression levels under hypoxic or PHD-inhibiting conditions. We discovered that

HELZ protein levels remain unaffected under these conditions. HELZ protein abundance is therefore not regulated by the PHDs and this finding suggests that even the existence of two conserved LxxLAP motifs cannot be taken as prerequisite for oxygen-dependent protein degradation or interaction with the PHDs. This point is supported by our discovery that, besides HIF-α and HELZ, only two other proteins contain two evolutionary conserved LxxLAP motifs namely CCR4-NOT transcription complex (CNOT1) and faciogenital dysplasia protein (FGD1) and none of them have yet been described in the context of oxygen-dependent regulation or PHD-interaction. Our observation is further supported by the finding of Huang and colleagues that, besides the hydroxylacceptor proline, none of the amino acids within the LxxLAP motif are required [176]. In another report, it has been shown that an amino acid substitution of the two leucines in the LxxLAP motif of a 20 residue motif had almost no effect on the substrate properties of the peptide [205]. In fact, of all the residues within the LxxLAP motif of HIF-α, only the hydroxylacceptor proline is relevant for substrate binding [206]. Interestingly, several different groups have shown that specific amino acids residues N-terminal or C-terminal of the LxxLAP motif are relevant for hydroxylation further supporting our notion that the existence of an LxxLAP motif alone is not enough for the prediction of oxygen-dependent regulation of a specific protein. For example, it has been shown that three naturally occurring mutations within HIF-2α (G537W, M535V and G537R) impair the binding of HIF-2α to PHD2 and VHL [207]. Another study showed that leucine at position 574 is crucial for VHL binding and VHL-mediated degradation of HIF-1α [177]. Furthermore, it has been shown that the substrate specificity of the PHDs is determined by a region relatively remote from the catalytic site [208]. These findings together with our observations suggest that the requirement of LxxLAP motifs for oxygen-dependent hydroxylation and protein degradation may be less important than originally thought.

Besides oxygen-dependent degradation, hydroxylation may have other, up to now unknown functions such as activation or suppression of protein activity. To examine this option we first investigated the yet unknown cellular function of HELZ. The resolution of the functional role of HELZ was initiated by the observation that overexpression of HELZ protein induces a heterologous luciferase reporter gene construct. The luciferase transcript levels remained unchanged suggesting that HELZ promotes protein translation. Methionine incorporation assay confirmed a stimulatory function for HELZ in protein translation. Since we were particularly interested in the

functionality of the HELZ LxxLAP motifs, we tested if the HELZ stimulatory function on translation involves the prolines within the HELZ LxxLAP motifs. However, mutation of one or both of the potential hydroxylacceptor prolines did not affect the HELZ stimulatory potential on translation. From this finding we can conclude that the prolines within the HELZ LxxLAP motifs are dispensable for HELZ function.

Regulation of protein translation is one way how cells control gene expression. This regulatory mechanism allows an instant response to a change in physiological conditions even in the absence of gene transcription. Protein translation is a four step process consisting of initiation, elongation, termination and ribosome recycling. Even though all steps are tightly regulated, translation initiation is the rate limiting step and the major site of regulation. The poly(A) binding protein (PABP) plays a key role in this regulation since it directly binds to the poly(A) tail of the mRNA resulting in the circularization of the mRNA due to the interaction of PABP with the eIF4F complex thereby facilitating translation initiation [8, 9]. Interaction with the poly(A) tail of mRNAs is established through the RNA recognition motifs (RRMs) within the N-terminal site of PABP [88]. Through its C-terminal site PABP associates with several of its interaction partners through their common PAM2 binding site [10]. These PABP interactors play a role in protein translation as well as mRNA stability [10]. Interestingly, HELZ harbours a PAM2 motif and the HELZ stimulatory role on translation may therefore be facilitated through its association with PABP. Indeed, our study confirmed that HELZ associates directly with PABP through its PAM2 motifs and indirectly with eIF4E probably via PABP. To our surprise the HELZ stimulatory effect on translation seemed to be independent of its interaction with PABP. However, we tested the possible involvement of PABP for the HELZ stimulatory activity on translation by mutating the PAM2-binding site of HELZ with PABP. It has been shown that PAIP1 interacts with PABP not only through its PAM2-binding site but also through another binding site termed PAM1, a motif that is composed of a 39 mainly acidic and hydrophobic amino acids [91]. It is interesting to note that the PAM1 site binds PABP with even a higher affinity than the PAM2 site [91]. Since the PAM1 binding site is relatively large and poorly defined (the physical basis of the PABP-PAM1 interaction site is not known) and seems to be different for Paip1 and Paip2 it is difficult to predict if HELZ contains a PAM1 site. It is therefore possible that HELZ interact with PABP at a site other than PAM2 and the interaction through

that site may be sufficient to promote HELZ function. We verified this idea by testing the HELZ-stimulatory role in translation in cells that have reduced PABP protein levels. Also these results show that the association of HELZ with PABP is dispensable for the HELZ-dependent induction of the luciferase reporter. However, we can not exclude that HELZ interacts with other members of the PABP family that may compensate for the lack of PABP or may even be the functional relevant interactor. In this context it has been shown that Tob1 interacts with PABP and iPABP via its PAM2 binding site [209]. It this study it has been shown that Tob1 interacts with iPABP and PABP and that Tob1 negatively regulates iPABP-mediated enhancement of translation of IL-2 mRNA. Inducible PABP received its name because it is rapidly upregulated in T cells and platelets upon their activation [101], [102]. The Genomics Institute of the Novartis Research Foundation (GNF) Atlas (http://biogps.gnf.org) documented that HELZ is relatively high expressed in lymphoid as well as myeloid cells compared to non-immune cells. This suggests that HELZ may play a role in iPABP function and therefore the HELZ-mediated induction of translation should be studied in the context of an iPABP interaction. In addition, since we tested HELZ function on an exogenously expressed luciferase construct it would be of great importance to study the function of HELZ on endogenous protein translation. Our global protein translation studies demonstrate a role of HELZ in endogenous protein translation and we also performed a polysomal profil analysis and observed that translation initiation is impaired in HELZ-deficient HeLa cells. It would be interesting to test if HELZ targets specific subsets of mRNAs harbouring subset-specific structures within their 5'-UTRs or, similar to Tob1, even mRNAs of specific genes. This could be tested by performing a gene array within the polyribosomes to test if certain mRNAs are differentially translated in HELZ-deficient cells. The interaction of HELZ with PABP or possibly iPABP can then be tested in the context of these mRNAs.

Nonetheless, our findings suggest that HELZ is member of the translation initiation complex that consists of PABP and other eukaryotic initiation factors such as eIF4E and bears a role in translation initiation.

Translation initiation factors play a vital role in cell proliferation and tumorigenesis. For example, stable expression of eIF4E in NIH3T3 and CHO cells enhances cellular proliferation and promotes growth in soft agar [210]. Overexpression of eIF4GI leads to tumor formation in nude mice [211]. eIF4A, a RNA helicase that is central for

optimal translation initiation, is found to be overexpressed in human melanoma cells and in primary hepatocellular carcinomas [212, 213]. DHX29 has been described to promote translation initiation and tumorigenesis and DDX3, a RNA helicase that is involved in translation initiation, has been described to promote cell growth [36, 214]. Based on these findings and our observation that HELZ functions in translation initiation, we thought to test if HELZ promotes cell proliferation. Indeed, our cell proliferation analysis demonstrated that HELZ promotes cell proliferation *in vitro*. It would be interesting to how this stimulating effect translates into the *in vivo* situation. It has been shown that HELZ is highly expressed in multiple tissues and organs within the developing embryo [62] and HELZ may therefore play a role in embryonic development. A HELZ *knock out* mouse model could give some answers on that question. Just recently, the ribosomal protein RPL38 has been found to specifically induce mRNA subsets of HOX genes during embryonic development [215]. It has been speculated, that RNA binding proteins may assist in this process [216]. It is very tempting to speculate an involvement of HELZ in this process. The previous observed increase in HELZ expression in the developing embryo [62] and the HELZ perinuclear expression [Hasgall et al., submitted for publication] would support such an idea. As mentioned, to find HELZ-specific target genes, a gene array in polyribosomes from HELZ-silenced versus wild-type cells should be performed. Such targets could then be studied in the context of embryonic development and ribosomal protein function. Also, the observed effect of HELZ on cell proliferation suggests a role of HELZ in tumorigenesis and this can be tested with an *in vivo* tumor mouse model testing tumor formation capacity of cells deficient in HELZ. A function of HELZ in tumorgenesis is supported by the observation that HELZ interacts with SMYD3, a methyltransferase that induces the expression of several oncogenes, and acts as a bridging protein between SMYD3 and RNA polymerase II [63].

The mTOR signaling pathway plays a central role in the control of cell growth and proliferation [217]. Most of the main targets of mTOR are components of the translation machinery, mainly initiation factors [218]. This includes eIF4B, eIF4G, eIF4E and S6K, the main kinase phosphorylating the ribosomal protein S6 that is a critical component of the 40S ribosomal subunit. Phosphorylation of the ribosomal protein S6 correlates positively with an increase in cell proliferation [219]. To test if the HELZ stimulatory effect on proliferation involves the mTOR signaling pathway, we analyzed the influence of HELZ on the phosphorylation of the ribosomal protein

S6. Strikingly, reduced HELZ protein levels in HeLa and HEK293 cells significantly correlated with reduced S6 phosphorylation. This observation cannot simply be explained by reduced S6 protein levels since they remain unaffected. A possible explanation could be that HELZ has an effect on the expression or the function of a kinase or even phosphatase upstream of S6. Our finding that HELZ plays a role in translation initiation would support such an idea. Precise mapping of the expression levels and phosphorylation state of all proteins that have an effect on the phosphorylation state of S6 would lead to the discovery of proteins that are affected by HELZ. The mRNA of these candidates could be then studied to reveal the mechanism on how HELZ affects their expression. In this regard a few RNA helicases or RNA binding proteins have been found to target specific subsets of mRNAs. For instance, eIF4A is responsible for unwinding of secondary structures of the 5’ UTR [220]. The RNA helicase A unwinds, similarly to eIF4A, secondary structures in the 5’ UTR but in a target specific manner through binding to complex 5′ post-transcriptional control elements [221]. The RNA helicase VASA also targets a specific subset of mRNAs by binding to the 3’ UTR and thereby facilitating translation initiation by recruitment of eIF5B in drosophila [222]. DHX29 targets mRNAs that have highly structure 5’ UTRs [37]. The RNA-binding protein embryonic-lethal abnormal vision (ELAV/Hu) stimulates the translation of mRNAs that contain an internal ribosomal entry site (IRES) [223].

Since S6 is mainly phosphorylated by the S6 kinase that is member of the mTOR signaling pathway we looked at some of the central members of the mTOR pathway namely mTOR, AKT and S6K, but there protein expression levels were not affected by HELZ. However, the mTOR pathway involved dozens of proteins and therefore we cannot exclude the involvement of the mTOR signaling pathway.

In summary, in the study presented herein we discovered that the putative RNA helicase HELZ plays a vital role in translation initiation and cellular proliferation. We showed that HELZ specifically modulates the phosphorylation state of the ribosomal protein S6. We found that HELZ contains two evolutionary conserved LxxLAP motifs, but these motifs are not functional since mutation of the prolines within the motifs does not affect HELZ protein levels or function. How HELZ mechanistically promotes S6 phosphorylation remains unknown, but may possibly involve the translation of specific subsets of mRNAs.

Part 7: References

1. Jankowsky, E. (2010) *RNA helicases at work: binding and rearranging.* Trends Biochem Sci.
2. Gebauer, F. and Hentze, M. W. (2004) *Molecular mechanisms of translational control.* Nat Rev Mol Cell Biol, **5**(10): p. 827-35.
3. Maxwell, P. H., *et al.* (1999) *The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis.* Nature, **399**(6733): p. 271-5.
4. Kallio, P. J., Wilson, W. J., O'Brien, S., Makino, Y. and Poellinger, L. (1999) *Regulation of the hypoxia-inducible transcription factor 1alpha by the ubiquitin-proteasome pathway.* J Biol Chem, **274**(10): p. 6519-25.
5. Salceda, S. and Caro, J. (1997) *Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes.* J Biol Chem, **272**(36): p. 22642-7.
6. Epstein, A. C., *et al.* (2001) *C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation.* Cell, **107**(1): p. 43-54.
7. Berra, E., Benizri, E., Ginouves, A., Volmat, V., Roux, D. and Pouyssegur, J. (2003) *HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1alpha in normoxia.* Embo J, **22**(16): p. 4082-90.
8. Tarun, S. Z., Jr. and Sachs, A. B. (1996) *Association of the yeast poly(A) tail binding protein with translation initiation factor eIF-4G.* Embo J, **15**(24): p. 7168-77.
9. Uchida, N., Hoshino, S., Imataka, H., Sonenberg, N. and Katada, T. (2002) *A novel role of the mammalian GSPT/eRF3 associating with poly(A)-binding protein in Cap/Poly(A)-dependent translation.* J Biol Chem, **277**(52): p. 50286-92.
10. Albrecht, M. and Lengauer, T. (2004) *Survey on the PABC recognition motif PAM2.* Biochem Biophys Res Commun, **316**(1): p. 129-38.
11. Abdelhaleem, M. (2005) *RNA helicases: regulators of differentiation.* Clinical Biochemistry, **38**(6): p. 499-503.
12. Anantharaman, V., Koonin, E. V. and Aravind, L. (2002) *Comparative genomics and evolution of proteins involved in RNA metabolism.* Nucleic Acids Res, **30**(7): p. 1427-64.
13. Matson, S. W., Bean, D. W. and George, J. W. (1994) *DNA helicases: enzymes with essential roles in all aspects of DNA metabolism.* Bioessays, **16**(1): p. 13-22.
14. Ray, B. K., Lawson, T. G., Kramer, J. C., Cladaras, M. H., Grifo, J. A., Abramson, R. D., Merrick, W. C. and Thach, R. E. (1985) *ATP-dependent unwinding of messenger RNA structure by eukaryotic initiation factors.* J Biol Chem, **260**(12): p. 7651-8.
15. Gorbalenya, A. E., Koonin, E. V., Donchenko, A. P. and Blinov, V. M. (1988) *A conserved NTP-motif in putative helicases.* Nature, **333**(6168): p. 22.

16. Gorbalenya, A. E. and Koonin, E. V. (1993) *Helicases: amino acid sequence comparisons and structure-function relationships.* Current Opinion in Structural Biology, **3**(3): p. 419-429.
17. Hall, M. C. and Matson, S. W. (1999) *Helicase motifs: the engine that powers DNA unwinding.* Mol Microbiol, **34**(5): p. 867-77.
18. Tuteja, N. and Tuteja, R. (2004) *Unraveling DNA helicases. Motif, structure, mechanism and function.* Eur J Biochem, **271**(10): p. 1849-63.
19. Bleichert, F. and Baserga, S. J. (2007) *The long unwinding road of RNA helicases.* Mol Cell, **27**(3): p. 339-52.
20. Czaplinski, K., Majlesi, N., Banerjee, T. and Peltz, S. W. (2000) *Mtt1 is a Upf1-like helicase that interacts with the translation termination factors and whose overexpression can modulate termination efficiency.* Rna, **6**(5): p. 730-43.
21. Walker, J. E., Saraste, M., Runswick, M. J. and Gay, N. J. (1982) *Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold.* Embo J, **1**(8): p. 945-51.
22. Smith, C. A. and Rayment, I. (1996) *Active site comparisons highlight structural similarities between myosin and other P-loop proteins.* Biophys J, **70**(4): p. 1590-602.
23. Pause, A. and Sonenberg, N. (1992) *Mutational analysis of a DEAD box RNA helicase: the mammalian translation initiation factor eIF-4A.* Embo J, **11**(7): p. 2643-54.
24. Jurnak, F. (1985) *Structure of the GDP domain of EF-Tu and location of the amino acids homologous to ras oncogene proteins.* Science, **230**(4721): p. 32-6.
25. Kadare, G. and Haenni, A. L. (1997) *Virus-encoded RNA helicases.* J Virol, **71**(4): p. 2583-90.
26. Leeds, P., Peltz, S. W., Jacobson, A. and Culbertson, M. R. (1991) *The product of the yeast UPF1 gene is required for rapid turnover of mRNAs containing a premature translational termination codon.* Genes Dev, **5**(12A): p. 2303-14.
27. Fairman-Williams, M. E., Guenther, U. P. and Jankowsky, E. (2010) *SF1 and SF2 helicases: family matters.* Curr Opin Struct Biol, **20**(3): p. 313-24.
28. Gorbalenya, A. E., Koonin, E. V. and Wolf, Y. I. (1990) *A new superfamily of putative NTP-binding domains encoded by genomes of small DNA and RNA viruses.* FEBS Lett, **262**(1): p. 145-8.
29. Hickman, A. B. and Dyda, F. (2005) *Binding and unwinding: SF3 viral helicases.* Curr Opin Struct Biol, **15**(1): p. 77-85.
30. Iyer, L. M., Leipe, D. D., Koonin, E. V. and Aravind, L. (2004) *Evolutionary history and higher order classification of AAA+ ATPases.* J Struct Biol, **146**(1-2): p. 11-31.
31. Klappacher, G. W., *et al.* (2002) *An induced Ets repressor complex regulates growth arrest during terminal macrophage differentiation.* Cell, **109**(2): p. 169-80.
32. Tanner, N. K. and Linder, P. (2001) *DExD/H box RNA helicases: from generic motors to specific dissociation functions.* Mol Cell, **8**(2): p. 251-62.
33. Kozak, M. (1978) *How do eucaryotic ribosomes select initiation regions in messenger RNA?* Cell, **15**(4): p. 1109-23.

34. Kozak, M. (1986) *Influences of mRNA secondary structure on initiation by eukaryotic ribosomes.* Proc Natl Acad Sci U S A, **83**(9): p. 2850-4.
35. Sonenberg, N. (1988) *Cap-binding proteins of eukaryotic messenger RNA: functions in initiation and control of translation.* Prog Nucleic Acid Res Mol Biol, **35**: p. 173-207.
36. Lee, C. S., Dias, A. P., Jedrychowski, M., Patel, A. H., Hsu, J. L. and Reed, R. (2008) *Human DDX3 functions in translation and interacts with the translation initiation factor eIF3.* Nucleic Acids Res, **36**(14): p. 4708-18.
37. Pisareva, V. P., Pisarev, A. V., Komar, A. A., Hellen, C. U. and Pestova, T. V. (2008) *Translation initiation on mammalian mRNAs with structured 5'UTRs requires DExH-box protein DHX29.* Cell, **135**(7): p. 1237-50.
38. Cheng, H., Dufu, K., Lee, C. S., Hsu, J. L., Dias, A. and Reed, R. (2006) *Human mRNA export machinery recruited to the 5' end of mRNA.* Cell, **127**(7): p. 1389-400.
39. Kapadia, F., Pryor, A., Chang, T. H. and Johnson, L. F. (2006) *Nuclear localization of poly(A)+ mRNA following siRNA reduction of expression of the mammalian RNA helicases UAP56 and URH49.* Gene, **384**: p. 37-44.
40. Tseng, S. S., Weaver, P. L., Liu, Y., Hitomi, M., Tartakoff, A. M. and Chang, T. H. (1998) *Dbp5p, a cytosolic RNA helicase, is required for poly(A)+ RNA export.* Embo J, **17**(9): p. 2651-62.
41. Culjkovic, B., Topisirovic, I., Skrabanek, L., Ruiz-Gutierrez, M. and Borden, K. L. (2006) *eIF4E is a central node of an RNA regulon that governs cellular proliferation.* J Cell Biol, **175**(3): p. 415-26.
42. Coller, J. M., Tucker, M., Sheth, U., Valencia-Sanchez, M. A. and Parker, R. (2001) *The DEAD box helicase, Dhh1p, functions in mRNA decapping and interacts with both the decapping and deadenylase complexes.* Rna, **7**(12): p. 1717-27.
43. Lykke-Andersen, J., Shu, M. D. and Steitz, J. A. (2000) *Human Upf proteins target an mRNA for nonsense-mediated decay when bound downstream of a termination codon.* Cell, **103**(7): p. 1121-31.
44. Chan, C. C., Dostie, J., Diem, M. D., Feng, W., Mann, M., Rappsilber, J. and Dreyfuss, G. (2004) *eIF4A3 is a novel component of the exon junction complex.* Rna, **10**(2): p. 200-9.
45. Nakajima, T., Uchida, C., Anderson, S. F., Parvin, J. D. and Montminy, M. (1997) *Analysis of a cAMP-responsive activator reveals a two-component mechanism for transcriptional induction via signal-dependent factors.* Genes Dev, **11**(6): p. 738-47.
46. Nakajima, T., Uchida, C., Anderson, S. F., Lee, C. G., Hurwitz, J., Parvin, J. D. and Montminy, M. (1997) *RNA helicase A mediates association of CBP with RNA polymerase II.* Cell, **90**(6): p. 1107-12.
47. Endoh, H., *et al.* (1999) *Purification and identification of p68 RNA helicase acting as a transcriptional coactivator specific for the activation function 1 of human estrogen receptor alpha.* Mol Cell Biol, **19**(8): p. 5363-72.
48. Fuller-Pace, F. V. (2006) *DExD/H box RNA helicases: multifunctional proteins with important roles in transcriptional regulation.* Nucleic Acids Res, **34**(15): p. 4206-15.
49. Knapp, G., Beckmann, J. S., Johnson, P. F., Fuhrman, S. A. and Abelson, J. (1978) *Transcription and processing of intervening sequences in yeast tRNA genes.* Cell, **14**(2): p. 221-36.

50. Grabowski, P. J., Seiler, S. R. and Sharp, P. A. (1985) *A multicomponent complex is involved in the splicing of messenger RNA precursors.* Cell, **42**(1): p. 345-53.
51. Frendewey, D. and Keller, W. (1985) *Stepwise assembly of a pre-mRNA splicing complex requires U-snRNPs and specific intron sequences.* Cell, **42**(1): p. 355-67.
52. Neubauer, G., King, A., Rappsilber, J., Calvio, C., Watson, M., Ajuh, P., Sleeman, J., Lamond, A. and Mann, M. (1998) *Mass spectrometry and EST-database searching allows characterization of the multi-protein spliceosome complex.* Nat Genet, **20**(1): p. 46-50.
53. Liu, Z. R. (2002) *p68 RNA helicase is an essential human splicing factor that acts at the U1 snRNA-5' splice site duplex.* Mol Cell Biol, **22**(15): p. 5443-50.
54. Wagner, J. D., Jankowsky, E., Company, M., Pyle, A. M. and Abelson, J. N. (1998) *The DEAH-box protein PRP22 is an ATPase that mediates ATP-dependent mRNA release from the spliceosome and unwinds RNA duplexes.* Embo J, **17**(10): p. 2926-37.
55. Burgess, S. M. and Guthrie, C. (1993) *A mechanism to enhance mRNA splicing fidelity: the RNA-dependent ATPase Prp16 governs usage of a discard pathway for aberrant lariat intermediates.* Cell, **73**(7): p. 1377-91.
56. Kressler, D., de la Cruz, J., Rojo, M. and Linder, P. (1998) *Dbp6p is an essential putative ATP-dependent RNA helicase required for 60S-ribosomal-subunit assembly in Saccharomyces cerevisiae.* Mol Cell Biol, **18**(4): p. 1855-65.
57. Daugeron, M. C. and Linder, P. (1998) *Dbp7p, a putative ATP-dependent RNA helicase from Saccharomyces cerevisiae, is required for 60S ribosomal subunit assembly.* Rna, **4**(5): p. 566-81.
58. de la Cruz, J., Kressler, D., Rojo, M., Tollervey, D. and Linder, P. (1998) *Spb4p, an essential putative RNA helicase, is required for a late step in the assembly of 60S ribosomal subunits in Saccharomyces cerevisiae.* Rna, **4**(10): p. 1268-81.
59. Kressler, D., de la Cruz, J., Rojo, M. and Linder, P. (1997) *Fal1p is an essential DEAD-box protein involved in 40S-ribosomal-subunit biogenesis in Saccharomyces cerevisiae.* Mol Cell Biol, **17**(12): p. 7283-94.
60. Nomura, N., *et al.* (1994) *Prediction of the coding sequences of unidentified human genes. I. The coding sequences of 40 new genes (KIAA0001-KIAA0040) deduced by analysis of randomly sampled cDNA clones from human immature myeloid cell line KG-1.* DNA Res, **1**(1): p. 27-35.
61. Nagai, H., *et al.* (2003) *Down-regulation in human cancers of DRHC, a novel helicase-like gene from 17q25.1 that inhibits cell growth.* Cancer Lett, **193**(1): p. 41-7.
62. Wagner, D. S., Gan, L. and Klein, W. H. (1999) *Identification of a differentially expressed RNA helicase by gene trapping.* Biochem Biophys Res Commun, **262**(3): p. 677-84.
63. Hamamoto, R., Furukawa, Y., Morita, M., Iimura, Y., Silva, F. P., Li, M., Yagyu, R. and Nakamura, Y. (2004) *SMYD3 encodes a histone methyltransferase involved in the proliferation of cancer cells.* Nat Cell Biol, **6**(8): p. 731-40.
64. Diehl, F., *et al.* (2010) *Cardiac deletion of Smyd2 is dispensable for mouse heart development.* PLoS One, **5**(3): p. e9748.

65. Davila Lopez, M. and Samuelsson, T. (2008) *Early evolution of histone mRNA 3' end processing.* Rna, **14**(1): p. 1-10.
66. Muthukrishnan, S., Both, G. W., Furuichi, Y. and Shatkin, A. J. (1975) *5'-Terminal 7-methylguanosine in eukaryotic mRNA is required for translation.* Nature, **255**(5503): p. 33-7.
67. Sonenberg, N., Morgan, M. A., Merrick, W. C. and Shatkin, A. J. (1978) *A polypeptide in eukaryotic initiation factors that crosslinks specifically to the 5'-terminal cap in mRNA.* Proc Natl Acad Sci U S A, **75**(10): p. 4843-7.
68. Svitkin, Y. V., Ovchinnikov, L. P., Dreyfuss, G. and Sonenberg, N. (1996) *General RNA binding proteins render translation cap dependent.* Embo J, **15**(24): p. 7147-55.
69. Pelletier, J. and Sonenberg, N. (1985) *Insertion mutagenesis to increase secondary structure within the 5' noncoding region of a eukaryotic mRNA reduces translational efficiency.* Cell, **40**(3): p. 515-26.
70. Jackson, R. J., Hellen, C. U. and Pestova, T. V. (2010) *The mechanism of eukaryotic translation initiation and principles of its regulation.* Nat Rev Mol Cell Biol, **11**(2): p. 113-27.
71. Grifo, J. A., Tahara, S. M., Morgan, M. A., Shatkin, A. J. and Merrick, W. C. (1983) *New initiation factor activity required for globin mRNA translation.* J Biol Chem, **258**(9): p. 5804-10.
72. Gingras, A. C., Raught, B. and Sonenberg, N. (1999) *eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation.* Annu Rev Biochem, **68**: p. 913-63.
73. Borman, A. M., Michel, Y. M. and Kean, K. M. (2000) *Biochemical characterisation of cap-poly(A) synergy in rabbit reticulocyte lysates: the eIF4G-PABP interaction increases the functional affinity of eIF4E for the capped mRNA 5'-end.* Nucleic Acids Res, **28**(21): p. 4068-75.
74. Sachs, A. B. and Davis, R. W. (1989) *The poly(A) binding protein is required for poly(A) shortening and 60S ribosomal subunit-dependent translation initiation.* Cell, **58**(5): p. 857-67.
75. Trachsel, H., Erni, B., Schreier, M. H. and Staehelin, T. (1977) *Initiation of mammalian protein synthesis. II. The assembly of the initiation complex with purified initiation factors.* J Mol Biol, **116**(4): p. 755-67.
76. Rheinberger, H. J., Sternbach, H. and Nierhaus, K. H. (1981) *Three tRNA binding sites on Escherichia coli ribosomes.* Proc Natl Acad Sci U S A, **78**(9): p. 5310-4.
77. Brands, J. H., Maassen, J. A., van Hemert, F. J., Amons, R. and Moller, W. (1986) *The primary structure of the alpha subunit of human elongation factor 1. Structural aspects of guanine-nucleotide-binding sites.* Eur J Biochem, **155**(1): p. 167-71.
78. Motorin Yu, A., Wolfson, A. D., Orlovsky, A. F. and Gladilin, K. L. (1988) *Mammalian valyl-tRNA synthetase forms a complex with the first elongation factor.* FEBS Lett, **238**(2): p. 262-4.
79. Nygard, O. and Nilsson, L. (1990) *Kinetic determination of the effects of ADP-ribosylation on the interaction of eukaryotic elongation factor 2 with ribosomes.* J Biol Chem, **265**(11): p. 6030-4.
80. Lee, C. C., Craigen, W. J., Muzny, D. M., Harlow, E. and Caskey, C. T. (1990) *Cloning and expression of a mammalian peptide chain release factor with sequence similarity to tryptophanyl-tRNA synthetases.* Proc Natl Acad Sci U S A, **87**(9): p. 3508-12.

81. Zhouravleva, G., Frolova, L., Le Goff, X., Le Guellec, R., Inge-Vechtomov, S., Kisselev, L. and Philippe, M. (1995) *Termination of translation in eukaryotes is governed by two interacting polypeptide chain release factors, eRF1 and eRF3.* Embo J, **14**(16): p. 4065-72.
82. Frolova, L., Le Goff, X., Zhouravleva, G., Davydova, E., Philippe, M. and Kisselev, L. (1996) *Eukaryotic polypeptide chain release factor eRF3 is an eRF1- and ribosome-dependent guanosine triphosphatase.* Rna, **2**(4): p. 334-41.
83. Hirashima, A. and Kaji, A. (1972) *Purification and properties of ribosome-releasing factor.* Biochemistry, **11**(22): p. 4037-44.
84. Pisarev, A. V., Hellen, C. U. and Pestova, T. V. (2007) *Recycling of eukaryotic posttermination ribosomal complexes.* Cell, **131**(2): p. 286-99.
85. Kolupaeva, V. G., Unbehaun, A., Lomakin, I. B., Hellen, C. U. and Pestova, T. V. (2005) *Binding of eukaryotic initiation factor 3 to ribosomal 40S subunits and its role in ribosomal dissociation and anti-association.* Rna, **11**(4): p. 470-86.
86. Sachs, A. B., Bond, M. W. and Kornberg, R. D. (1986) *A single gene from yeast for both nuclear and cytoplasmic polyadenylate-binding proteins: domain structure and expression.* Cell, **45**(6): p. 827-35.
87. Gorgoni, B. and Gray, N. K. (2004) *The roles of cytoplasmic poly(A)-binding proteins in regulating gene expression: a developmental perspective.* Brief Funct Genomic Proteomic, **3**(2): p. 125-41.
88. Deo, R. C., Bonanno, J. B., Sonenberg, N. and Burley, S. K. (1999) *Recognition of polyadenylate RNA by the poly(A)-binding protein.* Cell, **98**(6): p. 835-45.
89. Burd, C. G., Matunis, E. L. and Dreyfuss, G. (1991) *The multiple RNA-binding domains of the mRNA poly(A)-binding protein have different RNA-binding activities.* Mol Cell Biol, **11**(7): p. 3419-24.
90. Sachs, A. B., Davis, R. W. and Kornberg, R. D. (1987) *A single domain of yeast poly(A)-binding protein is necessary and sufficient for RNA binding and cell viability.* Mol Cell Biol, **7**(9): p. 3268-76.
91. Roy, G., De Crescenzo, G., Khaleghpour, K., Kahvejian, A., O'Connor-McCourt, M. and Sonenberg, N. (2002) *Paip1 interacts with poly(A) binding protein through two independent binding motifs.* Mol Cell Biol, **22**(11): p. 3769-82.
92. Kozlov, G., Menade, M., Rosenauer, A., Nguyen, L. and Gehring, K. (2010) *Molecular determinants of PAM2 recognition by the MLLE domain of poly(A)-binding protein.* J Mol Biol, **397**(2): p. 397-407.
93. Gorlach, M., Burd, C. G. and Dreyfuss, G. (1994) *The mRNA poly(A)-binding protein: localization, abundance, and RNA-binding specificity.* Exp Cell Res, **211**(2): p. 400-7.
94. Afonina, E., Stauber, R. and Pavlakis, G. N. (1998) *The human poly(A)-binding protein 1 shuttles between the nucleus and the cytoplasm.* J Biol Chem, **273**(21): p. 13015-21.
95. Gallie, D. R. (1998) *A tale of two termini: a functional interaction between the termini of an mRNA is a prerequisite for efficient translation initiation.* Gene, **216**(1): p. 1-11.
96. Bernstein, P., Peltz, S. W. and Ross, J. (1989) *The poly(A)-poly(A)-binding protein complex is a major determinant of mRNA stability in vitro.* Mol Cell Biol, **9**(2): p. 659-70.

97. Ivanov, P. V., Gehring, N. H., Kunz, J. B., Hentze, M. W. and Kulozik, A. E. (2008) *Interactions between UPF1, eRFs, PABP and the exon junction complex suggest an integrated model for mammalian NMD pathways.* Embo J, **27**(5): p. 736-47.
98. Fabian, M. R., *et al.* (2009) *Mammalian miRNA RISC recruits CAF1 and PABP to affect PABP-dependent deadenylation.* Mol Cell, **35**(6): p. 868-80.
99. Feral, C., Guellaen, G. and Pawlak, A. (2001) *Human testis expresses a specific poly(A)-binding protein.* Nucleic Acids Res, **29**(9): p. 1872-83.
100. Kleene, K. C., Wang, M. Y., Cutler, M., Hall, C. and Shih, D. (1994) *Developmental expression of poly(A) binding protein mRNAs during spermatogenesis in the mouse.* Mol Reprod Dev, **39**(4): p. 355-64.
101. Yang, H., Duckett, C. S. and Lindsten, T. (1995) *iPABP, an inducible poly(A)-binding protein detected in activated human T cells.* Mol Cell Biol, **15**(12): p. 6770-6.
102. Houng, A. K., Maggini, L., Clement, C. Y. and Reed, G. L. (1997) *Identification and structure of activated-platelet protein-1, a protein with RNA-binding domain motifs that is expressed by activated platelets.* Eur J Biochem, **243**(1-2): p. 209-18.
103. Blanco, P., Sargent, C. A., Boucher, C. A., Howell, G., Ross, M. and Affara, N. A. (2001) *A novel poly(A)-binding protein gene (PABPC5) maps to an X-specific subinterval in the Xq21.3/Yp11.2 homology block of the human sex chromosomes.* Genomics, **74**(1): p. 1-11.
104. Hünefeld, F. L. (1840) *Der Chemismus in der thierischen Organisation : Physiologisch-chemische Untersuchungen der materiellen Veränderungen, oder des Bildungslebens im thierischen Organismus; insbesondere des Blutbildungsprocesses, der Natur der Blut körperchen und ihrer Kernchen. Ein Beitrag zur Physiologie und Heilmittellehre.* Brockhaus: p. 160.
105. Hoppe-Seyler, F. (1866) *Über die oxydation in lebendem blute.* Med-chem Untersuch Lab., **1**: p. 133–140.
106. Viault, F. (1860) *Sur l'augmentation considérable du nombre des globules rouges dans le sang chez les habitants des hauts plateaux de 1'Amérique du Sud.* Comptes Rendus Hebdomaires des Seances de l'Académie des Sciences, **111**: p. 917–918.
107. Erslev, A. (1953) *Humoral regulation of red cell production.* Blood, **8**(4): p. 349-57.
108. Semenza, G. L. and Wang, G. L. (1992) *A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation.* Mol Cell Biol, **12**(12): p. 5447-54.
109. Wenger, R. H., Stiehl, D. P. and Camenisch, G. (2005) *Integration of oxygen signaling at the consensus HRE.* Sci STKE, **2005**(306): p. re12.
110. Hoffman, E. C., Reyes, H., Chu, F. F., Sander, F., Conley, L. H., Brooks, B. A. and Hankinson, O. (1991) *Cloning of a factor required for activity of the Ah (dioxin) receptor.* Science, **252**(5008): p. 954-8.
111. Wang, G. L. and Semenza, G. L. (1995) *Purification and characterization of hypoxia-inducible factor 1.* J Biol Chem, **270**(3): p. 1230-7.
112. Takahata, S., Sogawa, K., Kobayashi, A., Ema, M., Mimura, J., Ozaki, N. and Fujii-Kuriyama, Y. (1998) *Transcriptionally active heterodimer formation of an Arnt-like PAS protein, Arnt3, with HIF-1a, HLF, and clock.* Biochem Biophys Res Commun, **248**(3): p. 789-94.

113. Hirose, K., *et al.* (1996) *cDNA cloning and tissue-specific expression of a novel basic helix-loop-helix/PAS factor (Arnt2) with close sequence similarity to the aryl hydrocarbon receptor nuclear translocator (Arnt).* Mol Cell Biol, **16**(4): p. 1706-13.
114. Tian, H., McKnight, S. L. and Russell, D. W. (1997) *Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells.* Genes Dev, **11**(1): p. 72-82.
115. Wiesener, M. S., *et al.* (1998) *Induction of endothelial PAS domain protein-1 by hypoxia: characterization and comparison with hypoxia-inducible factor-1alpha.* Blood, **92**(7): p. 2260-8.
116. Hara, S., Hamada, J., Kobayashi, C., Kondo, Y. and Imura, N. (2001) *Expression and characterization of hypoxia-inducible factor (HIF)-3alpha in human kidney: suppression of HIF-mediated gene expression by HIF-3alpha.* Biochem Biophys Res Commun, **287**(4): p. 808-13.
117. Jiang, B. H., Rue, E., Wang, G. L., Roe, R. and Semenza, G. L. (1996) *Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1.* J Biol Chem, **271**(30): p. 17771-8.
118. Huang, L. E., Gu, J., Schau, M. and Bunn, H. F. (1998) *Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway.* Proc Natl Acad Sci U S A, **95**(14): p. 7987-92.
119. Kallio, P. J., Okamoto, K., O'Brien, S., Carrero, P., Makino, Y., Tanaka, H. and Poellinger, L. (1998) *Signal transduction in hypoxic cells: inducible nuclear translocation and recruitment of the CBP/p300 coactivator by the hypoxia-inducible factor-1alpha.* Embo J, **17**(22): p. 6573-86.
120. Luo, J. C. and Shibuya, M. (2001) *A variant of nuclear localization signal of bipartite-type is required for the nuclear translocation of hypoxia inducible factors (1alpha, 2alpha and 3alpha).* Oncogene, **20**(12): p. 1435-44.
121. Rankin, E. B. and Giaccia, A. J. (2008) *The role of hypoxia-inducible factors in tumorigenesis.* Cell Death Differ, **15**(4): p. 678-85.
122. Brieger, J., Weidt, E. J., Schirmacher, P., Storkel, S., Huber, C. and Decker, H. J. (1999) *Inverse regulation of vascular endothelial growth factor and VHL tumor suppressor gene in sporadic renal cell carcinomas is correlated with vascular growth: an in vivo study on 29 tumors.* J Mol Med, **77**(6): p. 505-10.
123. Takahashi, A., Sasaki, H., Kim, S. J., Tobisu, K., Kakizoe, T., Tsukamoto, T., Kumamoto, Y., Sugimura, T. and Terada, M. (1994) *Markedly increased amounts of messenger RNAs for vascular endothelial growth factor and placenta growth factor in renal cell carcinoma associated with angiogenesis.* Cancer Res, **54**(15): p. 4233-7.
124. Wiesener, M. S., *et al.* (2001) *Constitutive activation of hypoxia-inducible genes related to overexpression of hypoxia-inducible factor-1alpha in clear cell renal carcinomas.* Cancer Res, **61**(13): p. 5215-22.
125. Jewell, U. R., Kvietikova, I., Scheid, A., Bauer, C., Wenger, R. H. and Gassmann, M. (2001) *Induction of HIF-1alpha in response to hypoxia is instantaneous.* Faseb J, **15**(7): p. 1312-4.
126. Kallio, P. J., Pongratz, I., Gradin, K., McGuire, J. and Poellinger, L. (1997) *Activation of hypoxia-inducible factor 1alpha: posttranscriptional regulation and conformational change by recruitment of the Arnt transcription factor.* Proc Natl Acad Sci U S A, **94**(11): p. 5667-72.

127. Chilov, D., Camenisch, G., Kvietikova, I., Ziegler, U., Gassmann, M. and Wenger, R. H. (1999) *Induction and nuclear translocation of hypoxia-inducible factor-1 (HIF-1): heterodimerization with ARNT is not necessary for nuclear accumulation of HIF-1alpha.* J Cell Sci, **112 (Pt 8)**: p. 1203-12.
128. Mahon, P. C., Hirota, K. and Semenza, G. L. (2001) *FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity.* Genes Dev, **15**(20): p. 2675-86.
129. Lando, D., Peet, D. J., Gorman, J. J., Whelan, D. A., Whitelaw, M. L. and Bruick, R. K. (2002) *FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor.* Genes Dev, **16**(12): p. 1466-71.
130. Lando, D., Peet, D. J., Whelan, D. A., Gorman, J. J. and Whitelaw, M. L. (2002) *Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch.* Science, **295**(5556): p. 858-61.
131. Lee, F. S. and Percy, M. J. (2010) *The HIF Pathway and Erythrocytosis.* Annu Rev Pathol.
132. Bhattacharya, S., Michels, C. L., Leung, M. K., Arany, Z. P., Kung, A. L. and Livingston, D. M. (1999) *Functional role of p35srj, a novel p300/CBP binding protein, during transactivation by HIF-1.* Genes Dev, **13**(1): p. 64-75.
133. Jeong, J. W., *et al.* (2002) *Regulation and destabilization of HIF-1alpha by ARD1-mediated acetylation.* Cell, **111**(5): p. 709-20.
134. Feldser, D., Agani, F., Iyer, N. V., Pak, B., Ferreira, G. and Semenza, G. L. (1999) *Reciprocal positive regulation of hypoxia-inducible factor 1alpha and insulin-like growth factor 2.* Cancer Res, **59**(16): p. 3915-8.
135. Zelzer, E., Levy, Y., Kahana, C., Shilo, B. Z., Rubinstein, M. and Cohen, B. (1998) *Insulin induces transcription of target genes through the hypoxia-inducible factor HIF-1alpha/ARNT.* Embo J, **17**(17): p. 5085-94.
136. Agani, F. and Semenza, G. L. (1998) *Mersalyl is a novel inducer of vascular endothelial growth factor gene expression and hypoxia-inducible factor 1 activity.* Mol Pharmacol, **54**(5): p. 749-54.
137. Laughner, E., Taghavi, P., Chiles, K., Mahon, P. C. and Semenza, G. L. (2001) *HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression.* Mol Cell Biol, **21**(12): p. 3995-4004.
138. Hellwig-Burgel, T., Rutkowski, K., Metzen, E., Fandrey, J. and Jelkmann, W. (1999) *Interleukin-1beta and tumor necrosis factor-alpha stimulate DNA binding of hypoxia-inducible factor-1.* Blood, **94**(5): p. 1561-7.
139. Scharte, M., Han, X., Bertges, D. J., Fink, M. P. and Delude, R. L. (2003) *Cytokines induce HIF-1 DNA binding and the expression of HIF-1-dependent genes in cultured rat enterocytes.* Am J Physiol Gastrointest Liver Physiol, **284**(3): p. G373-84.
140. Bernardi, R., *et al.* (2006) *PML inhibits HIF-1alpha translation and neoangiogenesis through repression of mTOR.* Nature, **442**(7104): p. 779-85.
141. Richard, D. E., Berra, E., Gothie, E., Roux, D. and Pouyssegur, J. (1999) *p42/p44 mitogen-activated protein kinases phosphorylate hypoxia-inducible factor 1alpha (HIF-1alpha) and enhance the transcriptional activity of HIF-1.* J Biol Chem, **274**(46): p. 32631-7.
142. Hirota, K. and Semenza, G. L. (2001) *Rac1 activity is required for the activation of hypoxia-inducible factor 1.* J Biol Chem, **276**(24): p. 21166-72.

143. Thrash-Bingham, C. A. and Tartof, K. D. (1999) *aHIF: a natural antisense transcript overexpressed in human renal cancer and during hypoxia.* J Natl Cancer Inst, **91**(2): p. 143-51.
144. Makino, Y., Cao, R., Svensson, K., Bertilsson, G., Asman, M., Tanaka, H., Cao, Y., Berkenstam, A. and Poellinger, L. (2001) *Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression.* Nature, **414**(6863): p. 550-4.
145. Ivan, M., *et al.* (2001) *HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing.* Science, **292**(5516): p. 464-8.
146. Jaakkola, P., *et al.* (2001) *Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation.* Science, **292**(5516): p. 468-72.
147. Bruick, R. K. and McKnight, S. L. (2001) *A conserved family of prolyl-4-hydroxylases that modify HIF.* Science, **294**(5545): p. 1337-40.
148. Ivan, M., *et al.* (2002) *Biochemical purification and pharmacological inhibition of a mammalian prolyl hydroxylase acting on hypoxia-inducible factor.* Proc Natl Acad Sci U S A, **99**(21): p. 13459-64.
149. Lieb, M. E., Menzies, K., Moschella, M. C., Ni, R. and Taubman, M. B. (2002) *Mammalian EGLN genes have distinct patterns of mRNA expression and regulation.* Biochem Cell Biol, **80**(4): p. 421-6.
150. Metzen, E., *et al.* (2003) *Intracellular localisation of human HIF-1 alpha hydroxylases: implications for oxygen sensing.* J Cell Sci, **116**(Pt 7): p. 1319-26.
151. Soilleux, E. J., Turley, H., Tian, Y. M., Pugh, C. W., Gatter, K. C. and Harris, A. L. (2005) *Use of novel monoclonal antibodies to determine the expression and distribution of the hypoxia regulatory factors PHD-1, PHD-2, PHD-3 and FIH in normal and neoplastic human tissues.* Histopathology, **47**(6): p. 602-10.
152. Adluri, R. S., *et al.* (2010) *Disruption of HIF-Prolyl Hydroxylase-1 (PHD-1-/-) Attenuates Ex Vivo Myocardial Ischemia/Reperfusion Injury through HIF-1alpha Transcription Factor and its Target Genes in Mice.* Antioxid Redox Signal.
153. Tambuwala, M. M., *et al.* (2010) *Loss of prolyl hydroxylase-1 protects against colitis through reduced epithelial cell apoptosis and increased barrier function,* Gastroenterology, **139**(6): p. 2093-101.
154. Schneider, M., *et al. Loss or silencing of the PHD1 prolyl hydroxylase protects livers of mice against ischemia/reperfusion injury.* Gastroenterology, **138**(3): p. 1143-54 e1-2.
155. Jokilehto, T., Rantanen, K., Luukkaa, M., Heikkinen, P., Grenman, R., Minn, H., Kronqvist, P. and Jaakkola, P. M. (2006) *Overexpression and nuclear translocation of hypoxia-inducible factor prolyl hydroxylase PHD2 in head and neck squamous cell carcinoma is associated with tumor aggressiveness.* Clin Cancer Res, **12**(4): p. 1080-7.
156. Minamishima, Y. A., Moslehi, J., Bardeesy, N., Cullen, D., Bronson, R. T. and Kaelin, W. G., Jr. (2008) *Somatic inactivation of the PHD2 prolyl hydroxylase causes polycythemia and congestive heart failure.* Blood, **111**(6): p. 3236-44.
157. Takeda, K., Aguila, H. L., Parikh, N. S., Li, X., Lamothe, K., Duan, L. J., Takeda, H., Lee, F. S. and Fong, G. H. (2008) *Regulation of adult*

erythropoiesis by prolyl hydroxylase domain proteins. Blood, **111**(6): p. 3229-35.
158. Wax, S. D., Rosenfield, C. L. and Taubman, M. B. (1994) *Identification of a novel growth factor-responsive gene in vascular smooth muscle cells.* J Biol Chem, **269**(17): p. 13041-7.
159. Bishop, T., *et al.* (2008) *Abnormal sympathoadrenal development and systemic hypotension in PHD3-/- mice.* Mol Cell Biol, **28**(10): p. 3386-400.
160. McNeill, L. A., *et al.* (2005) *Hypoxia-inducible factor prolyl hydroxylase 2 has a high affinity for ferrous iron and 2-oxoglutarate.* Mol Biosyst, **1**(4): p. 321-4.
161. Hirsila, M., Koivunen, P., Gunzler, V., Kivirikko, K. I. and Myllyharju, J. (2003) *Characterization of the human prolyl 4-hydroxylases that modify the hypoxia-inducible factor.* J Biol Chem, **278**(33): p. 30772-80.
162. Stiehl, D. P., Wirthner, R., Koditz, J., Spielmann, P., Camenisch, G. and Wenger, R. H. (2006) *Increased prolyl 4-hydroxylase domain proteins compensate for decreased oxygen levels. Evidence for an autoregulatory oxygen-sensing system.* J Biol Chem, **281**(33): p. 23482-91.
163. Wang, G. L. and Semenza, G. L. (1993) *Desferrioxamine induces erythropoietin gene expression and hypoxia-inducible factor 1 DNA-binding activity: implications for models of hypoxia signal transduction.* Blood, **82**(12): p. 3610-5.
164. Schofield, C. J. and Ratcliffe, P. J. (2004) *Oxygen sensing by HIF hydroxylases.* Nat Rev Mol Cell Biol, **5**(5): p. 343-54.
165. Wolf, J. and Levy, I. J. (1954) *Treatment of sickle cell anemia with cobalt chloride.* AMA Arch Intern Med, **93**(3): p. 387-96.
166. Goldberg, M. A., Dunning, S. P. and Bunn, H. F. (1988) *Regulation of the erythropoietin gene: evidence that the oxygen sensor is a heme protein.* Science, **242**(4884): p. 1412-5.
167. Myllyla, R., Majamaa, K., Gunzler, V., Hanauske-Abel, H. M. and Kivirikko, K. I. (1984) *Ascorbate is consumed stoichiometrically in the uncoupled reactions catalyzed by prolyl 4-hydroxylase and lysyl hydroxylase.* J Biol Chem, **259**(9): p. 5403-5.
168. Knowles, H. J., Raval, R. R., Harris, A. L. and Ratcliffe, P. J. (2003) *Effect of ascorbate on the activity of hypoxia-inducible factor in cancer cells.* Cancer Res, **63**(8): p. 1764-8.
169. Nytko, K. J., Spielmann, P., Camenisch, G., Wenger, R. H. and Stiehl, D. P. (2007) *Regulated function of the prolyl-4-hydroxylase domain (PHD) oxygen sensor proteins.* Antioxid Redox Signal, **9**(9): p. 1329-38.
170. Baysal, B. E., *et al.* (2000) *Mutations in SDHD, a mitochondrial complex II gene, in hereditary paraganglioma.* Science, **287**(5454): p. 848-51.
171. Tomlinson, I. P., *et al.* (2002) *Germline mutations in FH predispose to dominantly inherited uterine fibroids, skin leiomyomata and papillary renal cell cancer.* Nat Genet, **30**(4): p. 406-10.
172. Pollard, P. J., *et al.* (2005) *Accumulation of Krebs cycle intermediates and over-expression of HIF1alpha in tumours which result from germline FH and SDH mutations.* Hum Mol Genet, **14**(15): p. 2231-9.
173. Selak, M. A., *et al.* (2005) *Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF-alpha prolyl hydroxylase.* Cancer Cell, **7**(1): p. 77-85.

174. Isaacs, J. S., *et al.* (2005) *HIF overexpression correlates with biallelic loss of fumarate hydratase in renal cancer: novel role of fumarate in regulation of HIF stability.* Cancer Cell, **8**(2): p. 143-53.
175. Masson, N., Willam, C., Maxwell, P. H., Pugh, C. W. and Ratcliffe, P. J. (2001) *Independent function of two destruction domains in hypoxia-inducible factor-alpha chains activated by prolyl hydroxylation.* Embo J, **20**(18): p. 5197-206.
176. Huang, J., Zhao, Q., Mooney, S. M. and Lee, F. S. (2002) *Sequence determinants in hypoxia-inducible factor-1alpha for hydroxylation by the prolyl hydroxylases PHD1, PHD2, and PHD3.* J Biol Chem, **277**(42): p. 39792-800.
177. Kageyama, Y., Koshiji, M., To, K. K., Tian, Y. M., Ratcliffe, P. J. and Huang, L. E. (2004) *Leu-574 of human HIF-1alpha is a molecular determinant of prolyl hydroxylation.* Faseb J, **18**(9): p. 1028-30.
178. Kuznetsova, A. V., Meller, J., Schnell, P. O., Nash, J. A., Ignacak, M. L., Sanchez, Y., Conaway, J. W., Conaway, R. C. and Czyzyk-Krzeska, M. F. (2003) *von Hippel-Lindau protein binds hyperphosphorylated large subunit of RNA polymerase II through a proline hydroxylation motif and targets it for ubiquitination.* Proc Natl Acad Sci U S A, **100**(5): p. 2706-11.
179. Abu-Farha, M., Niles, J. and Willmore, W. G. (2005) *Erythroid-specific 5-aminolevulinate synthase protein is stabilized by low oxygen and proteasomal inhibition.* Biochem Cell Biol, **83**(5): p. 620-30.
180. Hofer, T., Wenger, R. H., Kramer, M. F., Ferreira, G. C. and Gassmann, M. (2003) *Hypoxic up-regulation of erythroid 5-aminolevulinate synthase.* Blood, **101**(1): p. 348-50.
181. Cummins, E. P., *et al.* (2006) *Prolyl hydroxylase-1 negatively regulates IkappaB kinase-beta, giving insight into hypoxia-induced NFkappaB activity.* Proc Natl Acad Sci U S A, **103**(48): p. 18154-9.
182. Hanson, E. S., Rawlins, M. L. and Leibold, E. A. (2003) *Oxygen and iron regulation of iron regulatory protein 2.* J Biol Chem, **278**(41): p. 40337-42.
183. Wang, J., Chen, G., Muckenthaler, M., Galy, B., Hentze, M. W. and Pantopoulos, K. (2004) *Iron-mediated degradation of IRP2, an unexpected pathway involving a 2-oxoglutarate-dependent oxygenase activity.* Mol Cell Biol, **24**(3): p. 954-65.
184. Wang, J. and Pantopoulos, K. (2005) *The pathway for IRP2 degradation involving 2-oxoglutarate-dependent oxygenase(s) does not require the E3 ubiquitin ligase activity of pVHL.* Biochim Biophys Acta, **1743**(1-2): p. 79-85.
185. Koditz, J., Nesper, J., Wottawa, M., Stiehl, D. P., Camenisch, G., Franke, C., Myllyharju, J., Wenger, R. H. and Katschinski, D. M. (2007) *Oxygen-dependent ATF-4 stability is mediated by the PHD3 oxygen sensor.* Blood, **110**(10): p. 3610-7.
186. Nakayama, K., *et al.* (2004) *Siah2 regulates stability of prolyl-hydroxylases, controls HIF1alpha abundance, and modulates physiological responses to hypoxia.* Cell, **117**(7): p. 941-52.
187. Barth, S., *et al.* (2007) *The peptidyl prolyl cis/trans isomerase FKBP38 determines hypoxia-inducible transcription factor prolyl-4-hydroxylase PHD2 protein stability.* Mol Cell Biol, **27**(10): p. 3758-68.
188. Barth, S., Edlich, F., Berchner-Pfannschmidt, U., Gneuss, S., Jahreis, G., Hasgall, P. A., Fandrey, J., Wenger, R. H. and Camenisch, G. (2009) *Hypoxia-*

inducible factor prolyl-4-hydroxylase PHD2 protein abundance depends on integral membrane anchoring of FKBP38. J Biol Chem, **284**(34): p. 23046-58.

189. Mikhaylova, O., *et al.* (2008) *The von Hippel-Lindau tumor suppressor protein and Egl-9-Type proline hydroxylases regulate the large subunit of RNA polymerase II in response to oxidative stress.* Mol Cell Biol, **28**(8): p. 2701-17.
190. Martin, F., *et al.* (2005) *Copper-dependent activation of hypoxia-inducible factor (HIF)-1: implications for ceruloplasmin regulation.* Blood, **105**(12): p. 4613-9.
191. Bordoli, M. R., Stiehl, D. P., Borsig, L., Kristiansen, G., Hausladen, S., Schraml, P., Wenger, R. H. and Camenisch, G. (2010) *Prolyl-4-hydroxylase PHD2- and hypoxia-inducible factor 2-dependent regulation of amphiregulin contributes to breast tumorigenesis.* Oncogene.
192. Reed, R. and Hurt, E. (2002) *A conserved mRNA export machinery coupled to pre-mRNA splicing.* Cell, **108**(4): p. 523-31.
193. Nakielny, S. and Dreyfuss, G. (1999) *Transport of proteins and RNAs in and out of the nucleus.* Cell, **99**(7): p. 677-90.
194. Kohler, A. and Hurt, E. (2007) *Exporting RNA from the nucleus to the cytoplasm.* Nat Rev Mol Cell Biol, **8**(10): p. 761-73.
195. Eulalio, A., Behm-Ansmant, I. and Izaurralde, E. (2007) *P bodies: at the crossroads of post-transcriptional pathways.* Nat Rev Mol Cell Biol, **8**(1): p. 9-22.
196. Kedersha, N. L., Gupta, M., Li, W., Miller, I. and Anderson, P. (1999) *RNA-binding proteins TIA-1 and TIAR link the phosphorylation of eIF-2 alpha to the assembly of mammalian stress granules.* J Cell Biol, **147**(7): p. 1431-42.
197. Aprelikova, O., Pandolfi, S., Tackett, S., Ferreira, M., Salnikow, K., Ward, Y., Risinger, J. I., Barrett, J. C. and Niederhuber, J. (2009) *Melanoma antigen-11 inhibits the hypoxia-inducible factor prolyl hydroxylase 2 and activates hypoxic response.* Cancer Res, **69**(2): p. 616-24.
198. Shimomura, Y., Aoki, N., Ito, K. and Ito, M. (2003) *Gene expression of Sh3d19, a novel adaptor protein with five Src homology 3 domains, in anagen mouse hair follicles.* J Dermatol Sci, **31**(1): p. 43-51.
199. Buchan, J. R. and Parker, R. (2009) *Eukaryotic stress granules: the ins and outs of translation.* Mol Cell, **36**(6): p. 932-41.
200. Fonager, J., Beedholm, R., Clark, B. F. and Rattan, S. I. (2002) *Mild stress-induced stimulation of heat-shock protein synthesis and improved functional ability of human fibroblasts undergoing aging in vitro.* Exp Gerontol, **37**(10-11): p. 1223-8.
201. Camenisch, G., Tini, M., Chilov, D., Kvietikova, I., Srinivas, V., Caro, J., Spielmann, P., Wenger, R. H. and Gassmann, M. (1999) *General applicability of chicken egg yolk antibodies: the performance of IgY immunoglobulins raised against the hypoxia-inducible factor 1alpha.* Faseb J, **13**(1): p. 81-8.
202. Popowski, K., Eloranta, J. J., Saborowski, M., Fried, M., Meier, P. J. and Kullak-Ublick, G. A. (2005) *The human organic anion transporter 2 gene is transactivated by hepatocyte nuclear factor-4 alpha and suppressed by bile acids.* Mol Pharmacol, **67**(5): p. 1629-38.
203. Chilov, D., Hofer, T., Bauer, C., Wenger, R. H. and Gassmann, M. (2001) *Hypoxia affects expression of circadian genes PER1 and CLOCK in mouse brain.* Faseb J, **15**(14): p. 2613-22.

204. Balamurugan, K., *et al.* (2009) *Onconeuronal cerebellar degeneration-related antigen, Cdr2, is strongly expressed in papillary renal cell carcinoma and leads to attenuated hypoxic response.* Oncogene, **28**(37): p. 3274-85.
205. Li, D., Hirsila, M., Koivunen, P., Brenner, M. C., Xu, L., Yang, C., Kivirikko, K. I. and Myllyharju, J. (2004) *Many amino acid substitutions in a hypoxia-inducible transcription factor (HIF)-1alpha-like peptide cause only minor changes in its hydroxylation by the HIF prolyl 4-hydroxylases: substitution of 3,4-dehydroproline or azetidine-2-carboxylic acid for the proline leads to a high rate of uncoupled 2-oxoglutarate decarboxylation.* J Biol Chem, **279**(53): p. 55051-9.
206. Landazuri, M. O., Vara-Vega, A., Viton, M., Cuevas, Y. and del Peso, L. (2006) *Analysis of HIF-prolyl hydroxylases binding to substrates.* Biochem Biophys Res Commun, **351**(2): p. 313-20.
207. Furlow, P. W., Percy, M. J., Sutherland, S., Bierl, C., McMullin, M. F., Master, S. R., Lappin, T. R. and Lee, F. S. (2009) *Erythrocytosis-associated HIF-2alpha mutations demonstrate a critical role for residues C-terminal to the hydroxylacceptor proline.* J Biol Chem, **284**(14): p. 9050-8.
208. Villar, D., Vara-Vega, A., Landazuri, M. O. and Del Peso, L. (2007) *Identification of a region on hypoxia-inducible-factor prolyl 4-hydroxylases that determines their specificity for the oxygen degradation domains.* Biochem J, **408**(2): p. 231-40.
209. Okochi, K., Suzuki, T., Inoue, J., Matsuda, S. and Yamamoto, T. (2005) *Interaction of anti-proliferative protein Tob with poly(A)-binding protein and inducible poly(A)-binding protein: implication of Tob in translational control.* Genes Cells, **10**(2): p. 151-63.
210. Lazaris-Karatzas, A., Montine, K. S. and Sonenberg, N. (1990) *Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5' cap.* Nature, **345**(6275): p. 544-7.
211. Fukuchi-Shimogori, T., Ishii, I., Kashiwagi, K., Mashiba, H., Ekimoto, H. and Igarashi, K. (1997) *Malignant transformation by overproduction of translation initiation factor eIF4G.* Cancer Res, **57**(22): p. 5041-4.
212. Eberle, J., Krasagakis, K. and Orfanos, C. E. (1997) *Translation initiation factor eIF-4A1 mRNA is consistently overexpressed in human melanoma cells in vitro.* Int J Cancer, **71**(3): p. 396-401.
213. Shuda, M., *et al.* (2000) *Enhanced expression of translation factor mRNAs in hepatocellular carcinoma.* Anticancer Res, **20**(4): p. 2489-94.
214. Parsyan, A., *et al.* (2009) *The helicase protein DHX29 promotes translation initiation, cell proliferation, and tumorigenesis.* Proc Natl Acad Sci U S A, **106**(52): p. 22217-22.
215. Kondrashov, N., Pusic, A., Stumpf, C. R., Shimizu, K., Hsieh, A. C., Xue, S., Ishijima, J., Shiroishi, T. and Barna, M. (2011) *Ribosome-mediated specificity in hox mRNA translation and vertebrate tissue patterning.* Cell, **145**(3): p. 383-97.
216. Topisirovic, I. and Sonenberg, N. (2011) *Translational control by the eukaryotic ribosome.* Cell, **145**(3): p. 333-4.
217. Mamane, Y., Petroulakis, E., LeBacquer, O. and Sonenberg, N. (2006) *mTOR, translation initiation and cancer.* Oncogene, **25**(48): p. 6416-22.
218. Hay, N. and Sonenberg, N. (2004) *Upstream and downstream of mTOR.* Genes Dev, **18**(16): p. 1926-45.

219. Ruvinsky, I. and Meyuhas, O. (2006) *Ribosomal protein S6 phosphorylation: from protein synthesis to cell size.* Trends Biochem Sci, **31**(6): p. 342-8.
220. Rozen, F., Edery, I., Meerovitch, K., Dever, T. E., Merrick, W. C. and Sonenberg, N. (1990) *Bidirectional RNA helicase activity of eucaryotic translation initiation factors 4A and 4F.* Mol Cell Biol, **10**(3): p. 1134-44.
221. Hartman, T. R., Qian, S., Bolinger, C., Fernandez, S., Schoenberg, D. R. and Boris-Lawrie, K. (2006) *RNA helicase A is necessary for translation of selected messenger RNAs.* Nat Struct Mol Biol, **13**(6): p. 509-16.
222. Liu, N., Han, H. and Lasko, P. (2009) *Vasa promotes Drosophila germline stem cell differentiation by activating mei-P26 translation by directly interacting with a (U)-rich motif in its 3' UTR.* Genes Dev, **23**(23): p. 2742-52.
223. Kullmann, M., Gopfert, U., Siewe, B. and Hengst, L. (2002) *ELAV/Hu proteins inhibit p27 translation via an IRES element in the p27 5'UTR.* Genes Dev, **16**(23): p. 3087-99.

Part 8: Appendix

List of contributions to the publications and manuscripts

This thesis consists of the following original publications and manuscript and I contributed to it as indicated:

8.1 Publications

1. **Hasgall, P. A.**, Hoogewijs, D., Faza, M. B., Panse, V. G., Wenger, R. H. and Camenisch, G. (2011) The Putative RNA Helicase HELZ Interacts with Poly(A)-binding Protein and Promotes Translation Initiation and Ribosomal Protein S6 Phosphorylation. PLoS ONE. In revision.

    - Everything except the polysomal profil analysis

2. Barth, S., Edlich, F., Berchner-Pfannschmidt, U., Gneuss, S., Jahreis, G., **Hasgall, P. A.**, Fandrey, J., Wenger, R. H. and Camenisch, G. (2009) Hypoxia-inducible factor prolyl-4-hydroxylase PHD2 protein abundance depends on integral membrane anchoring of FKBP38. J Biol Chem **284**, 23046-23058

    - Cloning of construction used for FRET analysis in Figure 4C.

3. Barth, S., Nesper, J., **Hasgall, P. A.**, Wirthner, R., Nytko, K. J., Edlich, F., Katschinski, D. M., Stiehl, D. P., Wenger, R. H. and Camenisch, G. (2007) The peptidyl prolyl cis/trans isomerase FKBP38 determines hypoxia-inducible transcription factor prolyl-4-hydroxylase PHD2 protein stability. Mol Cell Biol **27**, 3758-3768

    - Figure 5A

8.2 Manuscripts

1. Hasgall, P. A., Wenger, R. H. and Camenisch. G. (2011) Characterization of novel HIF prolyl-4-hyrdroxylase 2-interacting proteins.

    - Everything

Curriculum vitae

PHILIPPE ARYE HASGALL
MANESSESTRASSE 105, 8045 ZURICH, SWITZERLAND
PHONE: ++41 (0)76 244 08 45 EMAIL: philippe.hasgall@gmail.com

PERSONAL INFORMATION

Nationality: Swiss
date of birth 26.01.1976

EDUCATION

PhD in Integrative Molecular Medicine June 2006 - June 2011
University of Zürich, Institute of Physiology, Zürich, Switzerland.
Laboratory of Professor Roland Wenger.

Master of Science in Immunopharmacology Sept 2003 - Feb 2006
Hebrew University, School of Pharmacy, Jerusalem, Israel.
Laboratory of Professor Francesca Levi-Schaffer.

Bachelor of Science Sep 2000 - Nov 2003
Hebrew University, Faculty of Science, Jerusalem, Israel.
Double major (Biology and Envriromental Science).

High School 1993 - 1998
Kantonsschule Kreuzlingen, Kreuzlingen, Switzerland.
(Type C, Science track)

LANGUAGES

German (native), French (fluent), English (fluent) and Hebrew (fluent)

INTERESTS & ACTIVITIES

- Member of the board of the nursing home "Schweizerisches Isreaelitisches Alters- und Pflegeheim" in Lengnau.
- Elderly care at elderly house at weekends.
- Sports (Biking, jogging, gym)

LIST OF PUBLICATIONS

1. **P. Hasgall et. al.** 2007 In Vitro Exposure To Cigarette Smoke Activates Eosinophils: Implications For Lung Inflammation. *The Internet Journal of Asthma, Allergy and Immunology*.;5(2).
2. **Barth S, Nesper J, Hasgall PA et al.** 2007 The peptidyl prolyl cis/trans isomerase FKBP38 determines hypoxia-inducible transcription factor prolyl-4-hydroxylase PHD2 protein stability. *Mol Cell Biol.*; 27(10):3758-68.
3. **Barth S et al**. 2009 Hypoxia-inducible factor prolyl-4-hydroxylase PHD2 protein abundance depends on integral membrane anchoring of FKBP38. *J Biol Chem*.; 284(34):23046-58.
4. **Hasgall PA et** al. 2009. The RNA helicase HELZ interacts with the poly(A) binding protein and promotes protein translation. PLoS ONE. In revision.

LIST OF PRESENTATIONS

Poster Presentations:

- International Eosinophil Society. Bern, Switzerland. 2005
- 2nd Symposium of the ZIHP, Zurich, Switzerland. 2006
- 3rd Symposium of the ZIHP, Zurich, Switzerland. 2007
- 4rd Symposium of the ZIHP, Zurich, Switzerland. 2008
- DPG (Deutsche Physiologische Gesellschaft), Cologne, Germany. 2008
- 5th Symposium of the ZIHP, Zurich, Switzerland. 2009
- 6th Symposium of the ZIHP, Zurich, Switzerland. 2010

Oral presentations:

- Gaslini International, Genova, Italy. 2005
- Institute of physiology, University of Zurich, Zurich, Switzerland. 2008
- ZIHP imMed PhD program retreat, Magglingen, Switzerland. 2008

Acknowledgments

This research project would not have been possible without the support of many people.

First of all I would like to thank Professor Roland Wenger for giving me the opportunity to perform my PhD thesis in his laboratory, for teaching me the value of critical thinking and for all the helpful discussions.

I would like to express my gratitude to my supervisor PD Dr Gieri Camenisch for his guidance, assistance and constant support.

I would like to thank my PHD committee member Professor Doerthe Katschinski and Dr. Thomas Gorr for joining my PhD committee and for helpful suggestions.

Many thanks go to Patrick Spielmann for constant technical assistance.

Thanks to Dr. Daniel Stiehl for giving me always good and useful ideas.

Thanks to Dr. Vikram Panse and Marius Faza for helping with the prolysomal profile analysis.

Thanks to all the Wenger Lab members for all the great moments.

Also, thanks to my brother Alexander for being a great friend and advisor.

I wish to express my love, gratitude and great appreciation to Sharon, my beloved wife and best friend. Without her understanding, encouragement and constant support it would not have been possible to perform my work.

Thanks to my three beautiful children Elisheva, Yael and Naftali for being such a great source of love and happiness.